UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

SEROLOGICAL SURVEILLANCE OF WEST NILE VIRUS AND MOLECULAR DIAGNOSTIC OF WEST NILE VIRUS, USUTU VIRUS, AVIAN INFLUENZA AND NEWCASTLE DISEASE VIRUS IN WILD BIRDS OF PORTUGAL

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ABSTRACT

SEROLOGICAL SURVEILLANCE OF WEST NILE VIRUS AND MOLECULAR DIAGNOSTIC OF WEST NILE VIRUS, USUTU VIRUS, AVIAN INFLUENZA AND NEWCASTLE DISEASE VIRUS IN WILD BIRDS OF PORTUGAL

The worldwide changes in the environment and climate of natural ecosystems detected in the last few decades have been responsible for the emergence of new infectious diseases in both animals and humans. This work focused on surveillance of four zoonotic pathogens, namely West Nile virus (WNV), Usutu virus (USUV), avian orthoavulavirus-1 (AOaV-1), also known as Newcastle disease virus (NDV), and influenza A virus (IAV) in wild birds of continental Portugal.

Blood and tissues samples from both live and dead birds (were collected in three wildlife rehabilitation centres of Portugal between 2018 and 2019: Wildlife Rehabilitation and Research Centre of Ria Formosa, Wildlife Rehabilitation Centre of Lisbon and University of Trás-os-Montes and Alto Douro Veterinary Teaching Hospital – Wildlife Rehabilitation Centre. Samples from a total of 192 animal were collected (82 *in vivo* and 110 *post-mortem*).

A total of one hundred and eighty-two samples were tested for WNV, USUV, IAV and for AOaV-1 by real time RT-PCR (RT-qPCR) or RT-PCR. AOaV-1 positive samples from two Eurasian collared doves (*Streptopelia decaocto*) (1.10% sample positivity) collected in the south of Portugal were sequenced, and their phylogenetic relationships analysed. Phylogenetic analysis confirmed that these sequences clustered with other AOaV-1 sequences from genotype XXI, subgenotype XXI.2.

Tissue samples were all negative for WNV, USUV and IAV.

Plasma samples were also tested for WNV antibodies by seroneutralization test. WNV neutralizing antibodies were detected in ten (13.70%) out of 73 samples namely: four *Buteo buteo*, two *Hieraaetus pennatus*, an *Accipiter nisus*, a *Aegypius monachus*, a *Circaetus gallicus*, and a *Ciconia ciconia*.

This study has established a baseline for future epidemiological studies of WNV and AOaV-1 in wild birds of continental Portugal. Further monitoring and epidemiological studies of both diseases in Portugal is advised, considering the threat that both diseases can pose to humans, animals and to the ecosystems themselves.

Keywords: West Nile virus, Usutu virus, Avian orthoavulavirus-1, Influenza A virus, wild birds

RESUMO

MONITORIZAÇÃO SEROLÓGICA DO VÍRUS DO NILO OCIDENTAL E DIAGNÓSTICO MOLECULAR DO VÍRUS DO NILO OCIDENTAL, VÍRUS USUTU, INFLUENZA AVIÁRIA E VÍRUS DA DOENÇA DE NEWCASTLE EM AVES SELVAGENS DE PORTUGAL

As profundas alterações ambientais e climáticas dos ecossistemas naturais que o mundo tem sofrido nas últimas décadas têm sido responsáveis pelo aparecimento de novas doenças infeciosas em animais e humanos. Este trabalho focou-se na monitorização de quatro agentes zoonóticos em aves selvagens de Portugal continental, nomeadamente vírus do Nilo Ocidental (WNV), vírus Usutu (USUV), orthoavulavirus-1 aviário, também conhecido como vírus da doença de Newcastle (NDV) e vírus influenza A (IAV).

Amostras de sangue e tecidos de animais vivos e mortos foram recolhidas entre 2018 e 2019 em três centros de recuperação de fauna selvagem em Portugal: Centro de Recuperação e Investigação de Animais Selvagens da Ria Formosa, Centro de Recuperação de Animais Silvestres de Lisboa e Centro de Recuperação de Animais Selvagens do Hospital Veterinário da UTAD. Foram recolhidas amostras de um total de 192 animais (82 *in vivo* e 110 *post-mortem*).

Um total de cento e oitenta e duas amostras foram testadas para a presença de WNV, USUV, IAV e AOaV-1 por RT-PCR em tempo real (RT-qPCR) e RT-PCR convencional. Duas amostras positivas de duas rolas turcas (*Streptopelia decaocto*) (1.10% positividade) recolhidas no sul de Portugal foram sequenciadas e as suas relações filogenéticas foram analisadas. A análise filogenética confirmou que estas sequências agrupam com estirpes de AOaV-1 do genótipo XXI, subgenótipo XXI.2.

Amostras de tecidos foram todas negativas para a presença de WNV, USUV e IAV.

Amostras de plasma foram testadas para a presença de anticorpos neutralizantes de WNV pelo teste da seroneutralização. Das 73 amostras, dez (13.70%) apresentavam anticorpos neutralizantes para WNV: quatro *Buteo buteo*, duas *Hieraaetus pennatus*, um *Accipiter nisus*, um *Aegypius monachus*, uma *Circaetus gallicus* e uma *Ciconia ciconia*.

Este estudo estabeleceu uma base para futuros estudos epidemiológicos sobre WNV e AOaV-1 em aves selvagens em Portugal continental. Aconselha-se a realização futura de outros estudos epidemiológicos e monitorizações, considerando a ameaça que ambas as doenças apresentam para humanos, animais e para os próprios ecossistemas.

Palavras-chave: vírus do Nilo Ocidental, vírus Usutu, orthoavulavirus-1 aviário, vírus influenza A, aves selvagens

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LIST OF ABBREVIATIONS

AOaV-1 - Avian orthoavulavirus-1

BLAST - Basic Local Alignment Search Tool

CI - Confidence interval

CNS - Central nervous system

CRAS-HVUTAD - Wildlife rehabilitation centre of the University of Trás-os-Montes e Alto Douro's Veterinary Teaching Hospital

Ct - Cycle threshold

DNA - Desoxyribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

EtOH - Ethanol

GTR – General time-reversible

HI - Hemagglutination Inhibition test

HPAIV - Highly pathogenic avian influenza virus

IAV - Influenza A virus

ICPI - Intracerebral Pathogenicity Index

ICTV - International Committee on Taxonomy of Viruses

IgG – Immunoglobulin G

IgM – Immunoglobulin M

Inf - Infinity

INIAV - Instituto Nacional de Investigação Agrária e Veterinária

LPAIV - Low pathogenic avian influenza virus

LxCRAS - Wildlife rehabilitation centre of Lisbon

mAb - Monoclonal antibody

mRNA - Messenger ribonucleic acid

ND – Newcastle disease

NDV - Newcastle disease virus

NUTS - Nomenclature of Territorial Units for Statistics

OIE - World Organisation for Animal Health

OR – Odds Ratio

ORF - Open reading frame

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PPMV-1 - Pigeon paramyxovirus type 1

RBC - Red blood cell

RdRp - RNA-dependent RNA polymerase

RIAS - Wildlife rehabilitation and research centre of Ria Formosa natural park

RNA - Ribonucleic acid

RNP - Ribonucleoprotein

RT-PCR - Reverse transcription-polymerase chain reaction

RT-qPCR - Quantitative reverse transcription-polymerase chain reaction

SNT – Seroneutralization test

TBE - Tris-Borate-EDTA

TCID - Tissue culture infective dose

USUV - Usutu virus

UTR - Untranslated region

vnND - Velogenic neurotropic Newcastle disease

vvND - Velogenic viscerotropic Newcastle disease

WNV – West Nile virus

LIST OF COMMON ENGLISH AND PORTUGUESE NAMES OF BIRD SPECIES

Latin name	English common name	Portuguese common name
Accipiter gentilis	Northern goshawk	Açor
Accipiter nisus	Eurasian sparrowhawk	Gavião-da-europa
Acridotheres cristatellus	Crested myna	Mainá-de-crista
Aegypius monachus	Cinereous vulture	Abutre-preto
Alectoris rufa	Red-legged partridge	Perdiz vermelha
Alopochen aegyptiaca	Egyptian goose	Ganso-do-Egipto
Anas crecca	Eurasian teal	Marrequinha-comum
Anas platyrynchos	Mallard	Pato-bravo
Apus apus	Common swift	Andorinhão-preto
Apus pallidus	Pallid swift	Andorinhão-pálido
Aquila adalberti	Spanish imperial eagle	Águia-imperial-ibérica
Aquila chrysaetos	Golden eagle	Águia-real
Aquila fasciatus	Bonelli's eagle	Águia-de-bonelli
Ardea cinerea	Grey heron	Garça-real
Asio flammeus	Short-eared owl	Coruja-do-nabal
Asio otus	Long-eared owl	Bufo-pequeno
Athene noctua	Little owl	Mocho-galego
Aythia fuligula	Tufted duck	Zarro-negrinha
Bubo bubo	Eurasian eagle owl	Bufo-real
Bubulbus ibis	Cattle egret	Garça-boieira
Buteo buteo	Common buzzard	Bútio-comum
Chroicocephalus ridibundus	Black-headed gull	Guncho-comum
Ciconia ciconia	White stork	Cegonha-branca
Circaetus gallicus	Short-toed snake eagle	Águia-cobreira
Columba livia	Rock dove	Pombo-das-rochas
Columba palombus	Common wood pidgeon	Pombo-trocaz
Copsychus sp.	Magpie robin	Pombo-pisco
Corvus corax	Common raven	Corvo-comum
Corvus corone	Carion crow	Gralha-preta
Erithacus rubecula	European robin	Pisco-de-peito-ruivo
Falco naumanni	Common kestrel	Peneireiro-das-torres
Falco peregrinus	Peregrine falcon	Falcão peregrino
Falco tinnunculus	Common kestrel	Peneireiro-vulgas
Fringilla coelebs	Common chaffinch	Tentilhão-comum
Fulica atra	Eurasian coot	Galeirão-comum
Gallinago gallinago	Common snipe	Narceja-comum
Gallinula chloropus	Common moorhen	Galinha-de-água
Gallus gallus domesticus	Chicken	Galinha
Garrulus glandarius	Eurasian jay	Gaio-comum
Hieraaetus pennatus	Booted eagle	Águia-calçada
Hirundo rustica	Barn swallow	Andorinha-das-chaminés
Larus audouinii	Audouin's gull	Gaivota-de-audouin
Larus fuscus	Lesser black-backed gull	Gaivota-de-asa-escura
Larus michahellis	Yellow-legged gull	Gaivota-de-patas-amarelas
Mareca strepera	Gadwall	Frisada

Latin name	English common name	Portuguese common name
Mergus merganser	Goosander	Merganso-grande
Milvus migrans	Black kite	Milhafre-preto
Milvus milvus	Red kite	Milhafre-real
Morus bassanus	Northern gannet	Ganso-patola
Netta ruffina	Red crested pochard	Pato-de-bico-vermelho
Nestor notabilis	Kea	Papagaio-da-Nova-Zelândia
Passer domesticus	House sparrow	Pardal-doméstico
Passer montanus	Tree sparrow	Pardal-montês
Pavo cristatus	Peafowl	Pavão-indiano
Pelecanus erythrorynchos	American white pelican	Pelicano-branco-americano
Perdix perdix	Grey partridge	Perdiz cinzenta
Pernis apivorus	European honey buzzard	Bútio-vespeiro
Phalacrocorax auritus	Double-crested cormorant	Corvo-marinho-de-orelhas
Phasianus colchicus	Common pheasant	Faisão-comum
Phoenicopterus sp.	Flamingo sp.	Flamingo sp.
Pica pica	Eurasian magpie	Pega-rabuda
Spatula clypeata	Northern shoveler	Pato-trombeteiro
Sterna hirundo	Common tern	Andorinha-do-mar-comum
Streptopelia decaocto	Eurasian collared dove	Rola-turca
Streptopelia turtur	European turtle dove	Rola-brava
Strix aluco	Tawny owl	Coruja-do-mato
Sturnus unicolor	Spotless starling	Estorninho-preto
Sylvia atricapila	Eurasian black cap	Toutinegra-de-barrete-preto
Taeniopygia guttata	Zebra finch	Mandarim
Turdus iliacus	Redwing	Tordo ruivo
Turdus merula	Common blackbird	Melro-preto
Turdus migratorius	American robin	Tordo-americano
Turdus philomelos	Song thrush	Tordo-comum
Turdus pilaris	Fieldfare	Tordo-zornal
Turdus torquatus	Ring ouzel	Melro-de-peito-branco
Tyto alba	Western barn owl	Coruja-das-torres

1. CURRICULAR TRAINEESHIP PERIOD ACTIVITIES

The authors 6th year traineeship was accomplished at the National Institute for Agrarian and Veterinary Research (INIAV, I.P.) from 11th February 2019 to 2nd August 2019, with a total amount of approximately 805 working hours.

The results allowed for the presentation of two posters:

1. "Serological surveillance of West Nile virus and molecular diagnostic of West Nile virus, Usutu virus, avian influenza and Newcastle disease virus in wild birds of Portugal" presented in the XXIV Encontro da Sociedade Portuguesa de Patologia Animal (June 15th-16th, 2019, Vila Real, Portugal) (Appendix 1);
2. "Newcastle disease virus (NDV) in two Eurasian Collared Doves (*Streptopelia decaocto*): A threat to European Turtle Dove (*Streptopelia turtur*) populations?" presented in the Wildlife & Game Management Innovation Summit (June 28th - 29th 2019, Lisbon, Portugal) (Appendix 2).

The work consisted in the molecular surveillance of West Nile virus, Usutu virus, avian orthoavulavirus-1 and influenza A virus and in the serological surveillance of West Nile virus in 192 samples (82 in vivo and 110 *post-mortem*) collected from wild birds in Portugal.

Most samples were collected before and during the traineeship (between June 11th, 2018 and June 9th, 2019) by the trainee in three wildlife rehabilitation centres of Portugal employing the sample collection and necropsy techniques learned during the Veterinary Medicine course. A small part of the samples was collected by the personnel of the wildlife rehabilitation centres.

The laboratory work involved sample preparation, extraction and purification of nucleic acids, RT-PCR and RT-qPCR, gel electrophoresis, DNA sequencing, viral isolation in embryonated eggs, hemagglutination tests, seroneutralization tests, cell culture techniques and phylogenetic and statistical analysis.

2. INTRODUCTION

Since the dawn of humanity birds have been a catching piece to the curious eye of the human being. Multiple factors have probably been contributing to this fascination: they rank as the world's most successful class of tetrapods, with approximately ten thousand living species; they are literally everywhere, with species living and breeding in most terrestrial habitats and on all seven continents; most of them have the ability to fly (in some cases distances of more than 80000 km per year) and their inter and intraspecific physical and behavioural differences makes each one of them a unique creature (Egevang et al. 2010). Since those times of simple curiosity, the relationship between birds and humans has grown hugely and to ends that one would have never guessed. We have portrayed them in mythology and literature, domesticated poultry, keep them as pets, use them in falconry, birdwatch and so on. Among all these relationships between birds and humans, there is one that is very interesting: the canary in the coalmine. Not because it gave origin to a common saying or because of their role in the world's mining history but because it portrays the concept of a sentinel species. In the 20th century, miners of Great Britain took caged canaries with them into coalmines to serve as a sentinel warning of the presence of toxic concentrations of gases such as carbon monoxide and methane. Canaries were used because they were more sensitive to carbon monoxide than humans and other studied animals, because they shared the same air as humans and finally because it was easy for a miner to detect the signs of carbon poisoning in an ill canary (Rabinowitz et al. 2009). Just as with the canary in the coalmine, zoonotic infectious diseases can be better detected and prevented when some animal species are used as sentinels, since the detection of diseases in animal populations may be a warning to the possibility of transmission to humans (Rabinowitz et al. 2009).

With the profound changes to the environment and climate of natural ecosystems, proposed as the major causes for the emergence of new infectious diseases in animals and humans, sentinel species for zoonotic infectious diseases are now, more than ever, a concept that should be employed by animal, human and environmental professionals, as part of the One Health paradigm, to give us important epidemiological information that can literally save human and animal lives (Rabinowitz et al. 2009). In this thesis wild birds were used as sentinel animals for the circulation of four zoonotic agents in continental Portugal: West Nile virus (WNV), Usutu virus (USUV), influenza A virus (IAV) and avian orthoavulavirus-1 (AOaV-1). Birds were used as sentinels for various reasons: (1) they are the primary vertebrate host of these diseases, (2) they are relatively numerous across our country and (3) it is relatively simple to have access to avian samples.

The objectives of this thesis were:

- to survey for evidence that WNV, USUV, IAV and AOaV-1 circulate in wild birds in continental Portugal;
- to attempt to associate the circulation of these pathogenic agents with the geographical location;
- to determine the host range of these viruses and see if there is any variability between bird order, species, age, sex, or migratory status.

2.1. The role of Portugal in the epidemiology of the studied avian infectious diseases

The epidemiology of avian infectious diseases is a complex function of biotic and abiotic factors. Migratory events, host ecology and, when in the presence of a vector-borne disease, vector ecology are some of the biotic factors that modulate infectious disease epidemiology, while climate, geography, bodies of water and other edaphoclimatic factors comprise the abiotic ones (Maclachlan and Dubovi 2016).

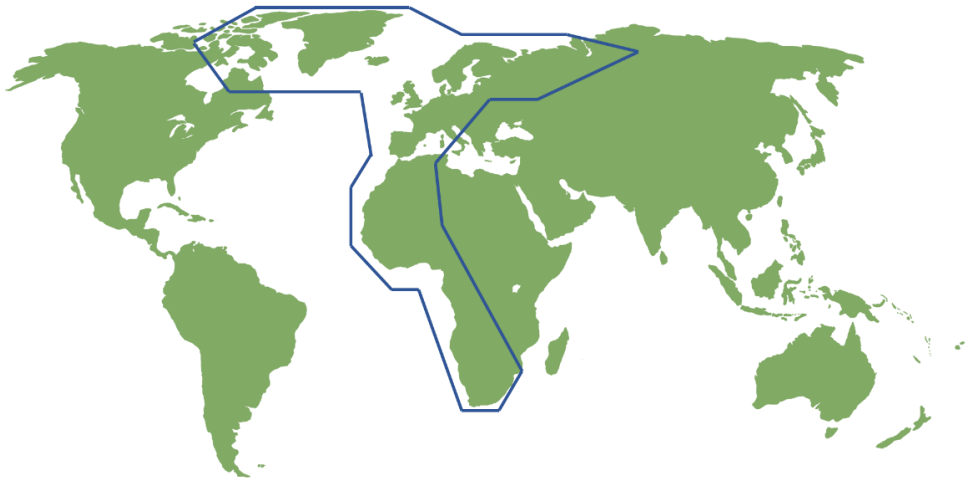
The migratory season occurs twice a year and involves the migration of billions of birds between continents in only a few weeks (Jourdain et al. 2007). During these events, birds can disperse zoonotic pathogens that pose a risk to both human and animal health (Reed et al. 2003). Migrating birds have specific destinies to which they migrate seasonally, following the same routes and passing through the same locations so it is possible to individualize these routes on the globe, which are called flyways (Equipa Atlas 2018). These flyways are the combination of regions and countries used as overlapping migratory systems that belong to different species and populations with their own migratory strategies and habitat specificities (Equipa Atlas 2018).

Portugal is in the East Atlantic flyway, a flyway that connects reproduction sites in the Arctic (from East Canada to Central Siberia), with reproduction and wintering sites at West Europe and wintering sites at South and West Africa, as presented in figure 1 (Equipa Atlas 2018). This flyway is currently used by 297 species of migratory birds and includes 75 countries, corresponding to an area larger than 45 000 000 Km^2 (Equipa Atlas 2018).

Some of these 297 species, like ducks, will end their journey in West Europe. Others, like waders, only make a stop in West Europe and then proceed their journey that will end in Africa (Equipa Atlas 2018). Some passerines and other terrestrial birds also come to Portugal during autumn and winter because the temperate and wet weather allows for good food availability (Equipa Atlas 2018). On the other hand, raptors that migrate to Africa need to pass through the strait of Gibraltar since they need thermal lifts to maintain their flight and since

crossing the Mediterranean Sea is nearly impossible for them (Equipa Atlas 2018). Considering the number of birds that use the East Atlantic flyway, one can conclude that continental Portugal is at a geographical location that can easily allow the introduction and transmission of avian infectious diseases.

Figure 1 – Main migratory flyways. The East Atlantic flyway, where Portugal is located, is represented in blue and connects reproduction sites in the Arctic with reproduction and wintering sites in West Europe and wintering sites in South and West Africa. Source: original, based on Equipa Atlas 2018.



Continental Portugal also has ecological conditions that can easily allow the introduction and transmission of IAV, AOaV-1, WNV and probably USUV. First, it has numerous wetlands, like marshes and ponds, where aquatic birds which migrate in the East Atlantic flyway can make a stop and rest (i.e. Tagus Estuary, Aveiro and Ria Formosa lagoons). These habitats aid the transmission of IAV and AOaV-1 because, in them, birds from different origins intermingle (Equipa Atlas 2018). As an example, the Tagus Estuary itself is thought to harbour about 50 000 aquatic birds during winter (Leitão et al. 1998).

Second, it seems to possess all the ecologic conditions for mosquitoes, the vectors of WNV, to thrive, such as many freshwater bodies that serve as breeding sites, and for the circulation and maintenance of WNV in its ecosystems (Formosinho et al. 2006). Likewise, it also seems to possess good weather conditions for the circulation and maintenance of WNV (Formosinho et al. 2006). Moreover, climatic changes can also have a role in the optimization of the conditions for the cycle of WNV, since high temperatures influence vector competence, accelerate viral replication in mosquitoes, increase their reproduction rates and prolong their breeding season (Lindgren et al. 2012; Paz et al. 2013; Tran et al. 2014). Long-term climatic changes can also affect vector and host activity (i.e. altering bird migratory routes, altering human land use) which could further affect the spatial-temporal distribution and prevalence of these diseases in the country (Semenza and Suk 2018). Furthermore, and considering that

USUV is also a flavivirus with similar ecological preferences and necessities to WNV, one can infer that, Portugal has all the ecological conditions for the circulation and maintenance of USUV in our ecosystems.

Finally, one cannot talk about the epidemiology of infectious diseases in the modern world without mentioning the way industrialisation and globalisation impacted it. In this highly interconnected world, various seemingly unrelated factors can contribute to the emergence of infectious diseases and Portugal is not an exception to the rule (Semenza and Suk 2018). For example, transport of goods and people through road, air or maritime route can also facilitate the dissemination of infectious diseases. Human built places, such as artificial lakes, wastewater treatment plants or sewage treatment plants can also favour the vector-host and/or host-host transmission of infectious diseases. For example, an Australian study found that water stabilization ponds are attractive to waterfowl because of their reliable water supply and high nutrient value, especially during droughts (Murray and Hamilton 2014). Other human constructions and activities can also facilitate the emergence and dissemination of avian infectious diseases. For instance, legal and illegal trade of pet birds or birds' products and live-poultry markets have been implied in the worldwide dissemination of numerous infectious diseases in the past (Karesh et al. 2005). Other human activities, such as light pollution, can also influence infectious disease risk, with one study referring that sparrows (*Passer domesticus*) exposed to artificial light at night maintained transmissible viral titres of WNV for two days longer than the control group, which may help to the dissemination of the disease (Kernbach et al. 2019).

2.2. West Nile virus

2.2.1. Characterization - Viral genome, structure, and life cycle

WNV is an arbovirus that belongs to the family *Flaviviridae* and to the genus *Flavivirus* that comprises 53 different species (Simmonds et al. 2017). It is classified within the Japanese Encephalitis serological complex of 9 genetically and antigenically related viruses (Kramer et al. 2008).

The WNV virion is spherical, enveloped, with approximately 40-60 nm in diameter and is encoded by an approximately 11 kb positive-sense single-stranded ribonucleic acid (RNA) genome (Mukhopadhyay et al. 2003, Kramer et al. 2008, Suthar et al. 2013). Mature virions contain a single copy of viral RNA consisting of a single open reading frame of approximately 11 kb with no polyadenylation tail at the 3' end. Both the 5' and 3' untranslated regions (UTR) form stem-loop structures that help in replication, transcription, translation, and packaging of the virus. The viral RNA is translated as a single polyprotein that is post- and translationally cleaved by viral and cellular proteases, resulting in three structural proteins, namely capsid

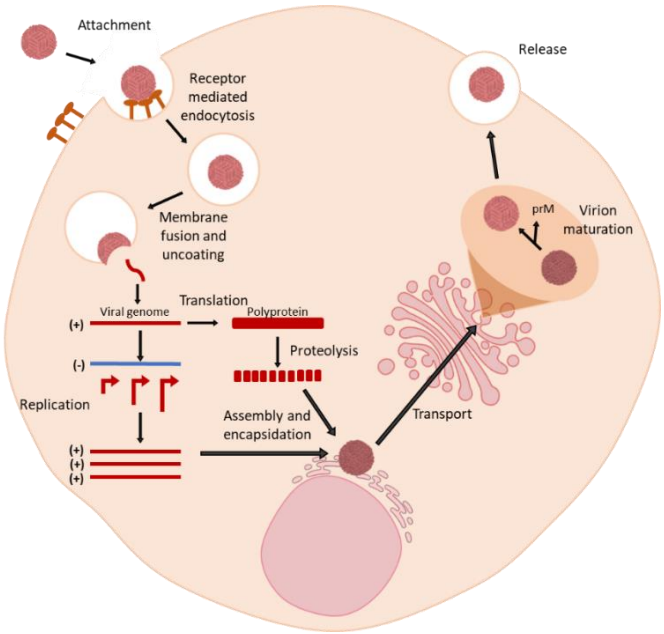
(C), pre-M/membrane (prM) and envelope (E) and seven non-structural (NS) proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Suthar et al. 2013; Chancey et al. 2015). The viral RNA is packaged within a ~50nm icosahedral capsid formed by the capsid protein that is surrounded by a host-derived lipid bilayer (Kramer et al. 2008; Colpitts et al. 2012; Chancey et al. 2015).

The structural proteins form the structure that encapsidates the viral RNA, and the non-structural proteins are multifunctional, playing critical roles in viral RNA synthesis and assembly (Kramer et al. 2008; Suthar et al. 2013; Brinton 2014). The NS1 protein is thought to play a role in regulating viral replication (Lindenbach and Rice 1997, Khromykh et al. 1999). NS3 has various enzymatic functions, serving as a viral serine protease (which cleaves the other non-structural proteins from the viral polyprotein) in association with NS2B, as an RNA helicase in association with NS4A and as a nucleoside triphosphatase in association with NS5 (Chancey et al. 2015). The NS5 protein is essential for viral replication because it contains RNA-dependent RNA polymerase (RdRp) activity in the C-terminal region and methyltransferase activity in the N-terminal region (Khromykh et al. 1999, Chancey et al. 2015). NS2A and NS4B have no known enzymatic functions (Youn et al. 2013, Chancey et al. 2015).

The WNV life cycle starts by the attachment of the virion to an still undefined cell surface receptor and entry to the cell by receptor-mediated endocytosis (Figure 2) (Suthar et al. 2013; Brinton 2014). The low pH environment within the endosomal vesicles triggers conformational changes of the envelope proteins that allows for the fusion of the virion with the endosomal membrane, uncoating and delivery of the infectious RNA genome into the cytoplasm (Suthar et al. 2013; Brinton 2014). Then, the genome is translated as a single polyprotein and viral serine protease complex (NS2B-NS3) and cellular proteases cleave this polyprotein into the three structural proteins and seven non-structural proteins (Suthar et al. 2013; Chancey et al. 2015). Then, the non-structural proteins that form the replication complex synthetise the intermediate negative-sense single strand RNA that serves as template for the synthesis of positive-sense single-strand RNA that will be used in the production of new virions (Colpitts et al. 2012; Suthar et al. 2013; Brinton 2014). The icosahedral capsid is formed by C protein which associates with RNA genome and mediates viral assembly in the endoplasmatic reticulum membranes (Markoff et al. 1997; Suthar et al. 2013; Brinton 2014; Chancey et al. 2015). During assembly, heterodimers of prM and E protein become embedded in the host-derived lipid bilayer of the virus, being exposed on the virion surface (Zhang et al. 2003, Chancey et al. 2015). The E protein mediates both binding of the receptor on the cell surface for viral entry and fusion with the host cell membrane. The prM protein protects the E protein from suffering irreversible conformational changes as the virion is secreted through acidified

sorting compartments (Kaufmann and Rossmann, 2011; Smit et al. 2011; Chancey et al. 2015). Then, the immature virions are transported through the Golgi and secretory pathway, where glycosylation of the viral E protein occurs and host cell furin-mediated cleavage of the protein prM to the mature M protein, thereby producing mature virions. The mature virions are released through exocytosis (Kramer et al. 2008; Suthar et al. 2013; Briton 2014; Chancey et al. 2015).

Figure 2 - Representation of WNV life cycle. Source: original, based on Suthar *et al*. 2013.



2.2.2. Genetic classification

WNV is a genetically diverse virus, with up to nine lineages proposed by various authors (Fall et al. 2017). Although, only lineages 1 and 2 have been associated with significant outbreaks in humans (Bakonyi et al. 2006; Chancey et al. 2015).

Lineage 1 is worldwide distributed and includes two clades: 1a and 1b. The clade 1a includes isolates from Africa, Europe, Middle East, Asia, and America. This lineage probably emerged in the beginning of the XX century in sub-Saharan or Northern Africa, spread towards other African countries and Europe, causing multiple small outbreaks in these places, and then, in the 1999, was imported to North America causing widespread human, bird and horse disease and mortality in there (Zehender et al. 2011, Chancey et al. 2015). Clade 1b includes the Australian Kunjin virus (KUNV) and is rarely associated with neurological disease in humans (Petersen and Rhoehrig 2001; Fall et al. 2017).

WNV lineage 2 was reported exclusively in Africa until 2004. However, since then, they have been implicated in multiple zoonotic outbreaks involving humans, horses and birds in central and eastern Europe (Bakonyi et al. 2006; Bakonyi et al. 2013; de Heus et al. 2020).

Besides lineage 1 and 2, there are lineages that are less widespread. Lineage 3, also known as Rabensburg virus, was isolated in the Czech Republic between 1997 and 1999, which have been demonstrated experimentally to only infect mosquitoes and their cells (Bakonyi et al. 2005; Hubálek et al. 2010; Aliota et al. 2012). Lineage 4 includes various isolates from Russia since 1988, comprising isolates from a tick, mosquitoes, and reptiles (Lvov et al. 2004). Lineage 5, often identified as a distinctive clade (clade 1c) of lineage 1, comprises isolates from India since 1955 (Bondre et al. 2007; May et al. 2011). Lineage 7, previously classified as a different virus, the Koutango virus from Africa comprises isolates from ticks and rodents (Fall et al. 2014). Other putative lineages have been proposed including a small group of isolates from Spain (lineage 6), an isolate found in a *Culex perfuscus* from Senegal (lineage 8) and an isolate from *Uranotaenia unguiculata* mosquitoes in Austria (lineage 9) (Vázquez et al. 2010; Pachler et al. 2014; Fall et al. 2017).

2.2.3. Hosts

WNV is transmitted in an enzootic cycle between birds and mosquitoes (Kilpatrick et al. 2007; Ferraguti et al. 2016). It has a complex eco-epidemiology that involves a wide range of vectors and enormous host diversity, being considered the most geographically widespread of all mosquito-borne flaviviruses (Ferraguti et al. 2016).

Over 300 bird species were reported as WNV positive only in the United States of America (U.S.A.), although only some of them are likely to be WNV reservoir hosts (CDC 2017). Some species of birds, like those belonging to the *Corvidae* family, are suggested to be extremely susceptible to WNV, since most individuals of this family become ill and even die from the infection (Komar et al. 2003). Other bird species, such as American robin (*Turdus migratorius)*, can develop high levels of viremia with a lower mortality rate, allowing for the virus maintenance and transmission to competent mosquito vectors, thus serving as an amplifying host (Komar et al. 2003; Chancey et al. 2015). Susceptibility to infection in birds has been associated with geographical range, body size, mating and breeding behaviour, migratory routes and with co-evolution with WNV or other antigenically related flaviviruses (Reisen et al. 2006; Reisen and Hahn 2007; Figuerola et al. 2008).

Beside birds, there are at least 30 other vertebrate species, including mammals, amphibians, and reptiles, which are susceptible to WNV infection (Chancey et al. 2015). Although, just a few non-avian vertebrates, such as eastern cottontail rabbits (*Sylvilagus floridanus*) and lake frogs (*Pelophylax ridibundus*), have been reported to develop viremia levels that are expected to support vector infection (Tiawsirisup et al. 2005; Van Der Meulen et al. 2005; Chancey et al. 2015). Humans and horses can also be infected by WNV and may suffer from serious disease or even death (Van Der Meulen et al. 2005). Although, both species

are incidental hosts because they do not develop viremia high enough to transmit the virus to competent mosquitoes (Martínez-De La Puente et al. 2018).

2.2.4. Vectors

At present, five European mosquito species have been demonstrated as competent vectors of WNV, namely *Culex pipiens*, *Cx. modestus*, *Cx. torrentium*, *Aedes albopictus*, and *A. detritus* (Balenghien et al. 2008; Fortuna et al. 2015; Blagrove et al. 2016; Jansen et al. 2019). In Portugal, WNV has been isolated from three mosquito species namely *Anopheles maculipennis, Cx. perexiguus,* and *Cx. pipiens* (Filipe and Pinto 1972; Parreira et al. 2007; Osório et al. 2012). Most authors suggest that *Cx. pipiens* is the most important vector in the epidemiology of WNV in Europe (Barros et al. 2011; Kilpatrick et al. 2007).

2.2.5. Transmission

The main transmission of WNV is through the bite of mosquitoes. The transmission cycle starts with competent mosquitoes feeding on an infected competent host, after which the virus replicates within the mosquito and is then transmitted to a susceptible host through salivary gland secretions when the mosquito bites it. WNV can also be transmitted directly through ingestion of infected animals or vectors and even by contact with cloacal or oral fluids from infected birds with high viremia (Komar et al. 2003; Pérez-Ramírez et al. 2014). In humans transplacental transmission and transmission through blood transfusion and organ transplantation have been documented (Iwamoto et al. 2003; Pealer et al. 2003; Gould and Fikrig 2004).

2.2.6. Clinical outcomes of avian infections

Highly susceptible species tend to die rapidly and may have few acute or non-observable lesions (Bertelsen et al. 2004; Wünschmann et al. 2004a). In less susceptible species, clinical disease is caused by the invasion of central nervous system (CNS), liver, spleen, kidney and heart (Steele et al. 2000; Gamino and Höfle 2013). Unspecific clinical signs normally include anorexia, depression, dehydration, and ruffled feathers. In 60% of infections can occur convulsions, in 30% of infections can occur ataxia and/or abnormal head posture and movements, and in up to 20% infections can present with tremors, uncoordinated flight, paresis and/or disorientation (Steele et al. 2000; Fitzgerald et al. 2003; D'Agostino and Isaza 2004; Wünschmann et al. 2004a). Impaired vision and blindness are relatively common in owls and raptors (Wünschmann et al. 2004a, 2005b; Gancz et al. 2006). Long-term sequelae have been detected in raptors, such as relapses of neurologic signs, abnormal molt and feather pulp abnormalities that can persist for up to 4 years (Nemeth et al. 2006; Nemeth et al. 2009).

Most birds eliminate the virus from their blood and body tissues within 2-3 weeks and remain clinically healthy during infection (Komar et al. 2003). Chronic infections for more than 12 months have only been reported in keas (*Nestor notabilis*) (Bakonyi et al. 2016).

2.2.7. Clinical outcomes of human infections

Approximately 80% of human infections are asymptomatic, while the remaining cases can have symptoms ranging from influenza-like symptoms to West Nile neuroinvasive disease (WNND). Influenza-like symptoms include fever, rash, arthralgia, and myalgia while neuroinvasive disease include severe symptoms that can lead to death such as meningoencephalitis, encephalitis, and polio-like flaccid paralysis (Jean et al. 2007; Hughes et al. 2007; Lindsey et al. 2010; Carson et al. 2012). Physical symptoms and/or cognitive deficits may persist over a year after infection (Klee et al. 2004; Sadek et al. 2010).

Risk factors in the development of severe disease include advanced age, immunosuppression, and chronic medical conditions such as diabetes and chronic renal failure (Jean et al. 2007; Hughes et al. 2007; Lindsey et al. 2010, 2012; Carson et al. 2012).

2.2.8. Clinical outcomes of horse infections

In horses, 20% of infections result in clinical disease, of which about 90% involve neurologic signs like ataxia, weakness, recumbency, muscle fasciculation, and high death rates (up to 30%) (Castillo-Olivares and Wood 2004).

2.2.9. Epidemiology

WNV was first described in 1937 from a febrile illness case in Uganda. Between the 1950s through the 1980s WNV only caused small outbreaks in Israel, France, Egypt, India and South Africa (Smithburn et al. 1940; Bernkopf et al. 1953; Jupp et al. 2001; Bondre et al. 2007; Balança et al. 2009). In 1957 the first outbreak of WNND was reported in Israel, however, it was only in 1999, when it crossed the Atlantic and reached New York City, U.S.A., that the virus started to cause widespread human, bird and horse disease and mortality cases (Zehender et al. 2011, Chancey et al. 2015). In the following years WNV spread to most U.S.A. territory, Canada and Mexico and even to South American countries (Maillo et al. 2008; Chancey et al. 2015). It is, nowadays, considered the most important cause of viral encephalitis worldwide (Chancey et al. 2015).

In Portugal, the first evidence of WNV circulation was documented in 1971 when the virus was isolated from mosquitoes (Filipe and de Andrade, 1990). Later, a serological survey conducted from 1999 to 2002 documented 11.9% (16/134) Flavivirus neutralizing antibodies in bird samples and 3.3% (3/91) in horse samples (Formosinho et al. 2006). Another study that analysed bird and horse samples collected during 2004-2010 detected WNV neutralizing

antibodies in 23 out of 116 samples of birds and 40 out of 1313 samples of horses (Barros et al. 2011). In the summer 2004, the first probable cases of human infection in Portugal were reported (Connell et al. 2004). The infection occurred in two tourists in the Algarve region, after which WNV was surveyed and detected in mosquitoes from the same region, with no further human cases detected (Esteves et al. 2004). Later, in 2010, another human case was detected in Algarve and in 2015 a WNND case was also detected in the region, being the first laboratory-confirmed human case that fully meets the European Union case definition for WNV.

In the Mediterranean basin, outbreaks of WNV infections in horses or birds in the last five years have been reported in Portugal (2016, 2017, 2018, 2019 and 2020) France (2017, 2018, 2019 and 2020), Italy (2016, 2017, 2018, 2019 and 2020), Spain (2016, 2017, 2018, 2019 and 2020) and Greece (2017, 2018, 2019 and 2020) (EC 2020; OIE 2020).

Most researchers think, that contrary to the situation in North America where most bird were naïve to WNV, in Europe WNV infections are usually asymptomatic in birds because the virus is endemic in the continent and so the numbers of WNV infection reported in both humans and animals was relatively low until 2004 (Hubálek and Halouzka 1999; Gray and Webb 2014). However, in 2004 lineage 2 WNV was detected in Hungary in a northern goshawk (*Accipiter gentilis*) with neurological signs and in the following years, it has spread within Eastern and Central Southern Europe, becoming endemic in this part of Europe (Bakonyi et al. 2006; Hernández-Triana et al. 2014). Since then, there has been an unprecedented rise in the number of WNV reported cases, of both lineages, with researchers pointing out to the possibility of an increase in the virus virulence (Gray and Webb 2014).

2.2.10. Diagnosis

Clinical presentation of WNV infection is highly diverse and similar to other flaviviruses, making laboratorial diagnosis essential (Dauphin and Zientara 2007). Molecular methods, such as quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays, are a common tool to detect the presence of viral RNA in hosts. When the presence of viral RNA cannot be demonstrated by molecular assays, serological assays can be used to demonstrate that the host has, in some moment of its life, contacted with the virus. The existence of a specific immunoglobulin M (IgM) humoral response is considered an indicator of early-stage infection and serum, plasma, or cerebrospinal fluid (CSF) are the samples of choice. Serological cross-reactivity among flaviviruses is a major diagnostic challenge when using immunoglobulin G (IgG) enzyme-Linked Immunosorbent (ELISA) assays, significantly lowering the specificity of these tests (Pierson and Diamond 2008). The use of seroneutralization test (SNT) minimizes this cross-reactivity between flaviviruses, being

especially important in areas where related flaviviruses co-circulate, preventing overestimates of WNV neutralization antibodies presence (Beck et al. 2013).

2.3. USUTU virus

2.3.1. Characterization - viral genome, structure, and life cycle

USUV is an arbovirus that belongs to the *Flaviviridae* family, genus *Flavivirus*. USUV belongs to the same antigenic complex of WNV, the Japanese encephalitis serocomplex (Llorente et al. 2013). The USUV virion is small, spherical with a lipid envelope derived from the host cell membrane and has approximately 40-60 nm in diameter (Gaibani and Rossini 2017). Its genomic organization has a similar structure to other flavivirus (Bakonyi et al. 2003). The virion contains a single positive-sense RNA genome of approximately 11 kb in length with no polyadenylation tail at the 3' end. The genome consists of RNA genome with a 5' cap structure, a unique open reading frame (ORF) and two UTRs (Gaibani and Rossini 2017). The predicted ORF is translated into a polyprotein of 3434 amino acids that is post-translationally processed into three structural proteins, C, E, and prM, and seven non-structural proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Bakonyi et al. 2014; Saiz and Blazquez 2017). The C protein forms the central core of the virion and is associated to the viral RNA. The E protein mediates binding to the host cells and promotes viral entry into the cells. The prM protein is necessary for virion assembly and maturation by assisting envelope folding (Li et al. 2008; Smit et al. 2011). The non-structural proteins have different roles during the infection and their function is deduced based on similarity with other flaviviruses (Murray et al. 2008). NS1, is thought to play a role on the replication of viral genome (Watterson et al. 2016). The NS2B and NS4A are needed for virus assemblage (Pauli et al. 2014). NS3 protein encodes for viral serine protease (in association with NS2B), helicase, nucleoside triphosphatase and RNA triphosphatase and NS5 protein encodes for a methyltransferase at the N-terminal and an RNA-dependent RNA polymerase at the C-terminal (Pauli et al. 2014; Londono-Renteria et al. 2016). NS2A and NS4B have no known enzymatic functions (Leung et al. 2008, Youn et al. 2013, Chancey et al. 2015).

It is assumed that the USUV replication cycle is similar to those of the other flaviviruses, such as the WNV replication cycle described earlier (Saiz and Blazquez 2017).

2.3.2. Genetic classification

USUV is grouped in two major groups, African and European (Engel et al. 2016). Some authors have proposed three distinct lineages in the African group (Africa 1 to 3) and five different lineages in the European group (Europe 1 to 5) based on phylogenetic analysis of the NS5 gene (Cadar et al. 2017). The African group comprises strains isolated from the Central African Republic, Senegal, Germany, France, Netherlands, and Belgium (Nikolay et al. 2013;

Gaibani and Rossini 2017). The European group comprises isolates from Austria, Hungary, Switzerland, Senegal, Italy, Czech Republic, Germany, Belgium, and France (Gaibani et al. 2013; Engel et al. 2016; Cadar et al. 2017; Gaibani and Rossini 2017; Garigliany et al. 2017; Grottola et al. 2017). Genome identity among all isolates is higher than 94%, with exception of the strain of Africa 1 lineage that as a nucleotide identity of 78.3% (Nikolay et al. 2013; Bakonyi et al. 2014).

2.3.3. Hosts

USUV was detected in at least 62 species and 13 orders of birds (Gaibani and Rossini 2017; Clé et al. 2019). Some authors have suggested that birds from the Turdus genus, such as common blackbird (*Turdus merula*) and song thrush (*Turdus philomelos*) are more susceptible to this virus and appear to suffer the highest mortality rates (Weissenböck et al. 2002; Höfle et al. 2013).

It has also been detected in other vertebrate species such as bats, namely, *Pipistrellus pipistrellus*, horses, dogs, and wild ruminants such as red deer (*Cervus elaphus*), fallow deer (*Dama dama*), european mouflon (*Ovis aries musimon*) and roe deer (*Capreolus capreolus*) (Durand et al. 2012; Barbic et al. 2013; Cadar et al. 2013; García-Bocanegra et al. 2016). The consequences of the disease to these species have not been well assessed.

2.3.4. Vectors

Regarding vectors, it has been detected in eight mosquito species belonging to the genus Aedes, Anopheles, Culex, Culiseta, Ochlerotatus, Coquillettidia and Mansonia (Nikolay et al. 2011; Nikolay 2015). *Culex pipiens* is considered the main vector of USUV, while *Cx. neavei* is hypothesised to be involved in the sylvatic transmission in Africa (Calzolani et al. 2010; Nikolay et al. 2011; Fros et al. 2015).

2.3.5. Transmission

The natural life cycle of USUV is identical to WNV, involving mosquitoes as vectors and birds as main hosts, with humans being considered incidental hosts (Gaibani and Rossini 2017).

2.3.5. Clinical outcomes of avian infections

Normally the principal sign of USUV infection in wild birds of Europe has been the occurrence of mortality events. In captivity, the most reported clinical signs in birds are nonspecific signs, such as prostration, ruffled plumage and weight lost, and neurological signs such as disorientation, ataxia and seizures (Weissenböck et al. 2002; Steinmetz et al. 2011; Clé et al. 2019).

Gross lesions observed in dead birds normally consist of general congestion, hepatomegaly, and splenomegaly. Microscopically, encephalitis, myocardial degeneration and inflammation and necrosis of the liver, spleen and kidney are normally found (Chvala et al. 2004; Bakonyi et al. 2007; Manarolla et al. 2010).

2.3.6. Clinical outcomes of human infections

Like WNV, infections can be asymptomatic or present with mild symptoms such as rash, fever and headache (Pecorari et al. 2009; Bakonyi et al. 2017). In some cases, neuroinvassivenes occurs and patients present encephalitis, meningoencephalitis and/or idiopathic facial paralysis (Cadar et al. 2017; Simonin et al. 2018).

2.3.6. Epidemiology

A recent study by Engel et al. (2016) hypothesized that USUV emerged in Africa at the beginning of 16th century being exclusively limited to the African continent until the XX century (Nikolay et al. 2011; Engel et al. 2016).

USUV was first discovered in 1959 in South Africa from a mosquito species *Culex univittatus* (McIntosh 1985). Since then, it has been detected in other African countries such as Burkina Faso, Central African Republic, Ivory Coast, Kenya, Morocco, Nigeria, Senegal, Tunisia and Uganda (Nikolay et al. 2011; Durand et al. 2016; Mossel et al. 2017).

The first evidence of circulation of USUV in Europe dates to 2001, when multiple resident bird species died in Austria due to USUV infection (Weissenböck et al. 2002). Although, retrospective analyses of archived tissue samples from common blackbirds (*T. merula*) dating back to 1996 from Tuscany, Italy, have provided evidence that USUV was present in Europe at least since that year (Mani et al. 1998; Weissenböck et al. 2013).

In the following years, USUV has been isolated from mosquitoes, birds, bats and/or humans in other European countries namely Belgium, Croatia, Czech Republic, England, France, Germany, Greece, Hungary, The Netherlands, Poland, Serbia, Slovakia, Spain and Switzerland (Bakonyi et al. 2007; Linke et al. 2007; Busquets et al. 2008; Manarolla et al. 2010; Steinmetz et al. 2011; Chaintoutis et al. 2014; Garigliany et al. 2014; Hubálek et al. 2014; Vilibic-Cavlek et al. 2014; Lecollinet et al. 2015; Rijks et al. 2016; Bażanów et al. 2018; Csank et al. 2018; Kemenesi et al. 2018; Folly et al. 2020). This data suggests a continuous geographical spread of the virus, colonisation of new ecological niches and even that the virus has become endemic in some European countries (Clé et al. 2019). To the author's knowledge, USUV circulation proof has not been yet reported in Portugal.

Apart from Europe and Africa, USUV was only detected in *Culex sp.* mosquitoes in Israel during 2014-2015 (Mannasse et al. 2017).

2.3.7. Diagnosis

USUV diagnostic is similar to WNV. Infection can be detected directly by molecular methods or viral isolation in cell culture (for example in Vero cells), or indirectly, through serological methods, such as ELISA or immunofluorescence tests (Zannoli and Sambri 2019). In the same way that occurs in WNV, a positive result must be confirmed with more specific tests, such as SNT, to diminish the cross-reactivity with antibodies against other flaviviruses (Zannoli and Sambri 2019).

2.4. Avian orthoavulavirus type-1 (AOaV-1)

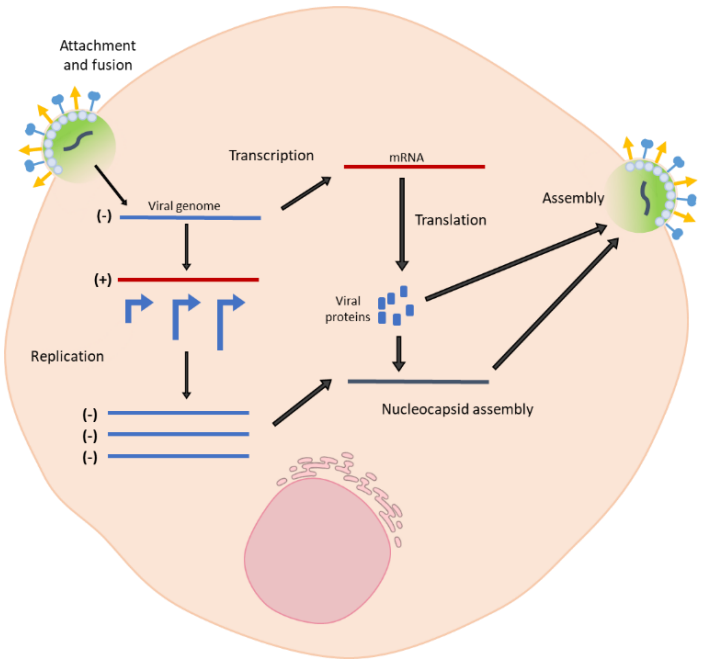
2.4.1. Characterization – viral genome, structure, and life cycle

AOaV-1 also known as Newcastle disease virus (NDV), avian avulavirus 1 (AAvV-1) or avian paramyxovirus 1 (APMV-1) is a member of the family *Paramyxoviridae* (Dimitrov et al. 2019). AOaV-1 is an enveloped, non-segmented, negative-sense, single-stranded RNA virus with a genome with approximately 15200 nucleotides (Hines and Miller 2012; Maclachlan and Dubovi 2016). Virions are pleomorphic, with filamentous and spherical forms occurring, with 150-300 nm in diameter and have an envelope covered with large spikes (Lambs and Parks 2013; Maclachlan and Dubovi 2016). Its genome encodes six proteins: nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), and the large polymerase (L) protein (Hines and Miller 2012; Maclachlan and Dubovi 2016). In addition, during P gene transcription, one additional, non-structural protein (V) is produced by means of mRNA editing, but its function is yet to be completely defined (Steward et al. 1993; Lambs and Parks 2013; Maclachlan and Dubovi 2016). The P and L proteins form the RdRp and are associated with the N protein-encapsidated viral RNA forming the ribonucleoprotein (RNP) complex. All these proteins are required for viral synthesis (Hines and Miller 2012; Maclachlan and Dubovi 2016). The M protein is the most abundant in the virion. It interacts with the lipid envelope, the F protein and the RNP complex, having an essential role in the assembly of mature virions, by providing a structural link between the envelope glycoproteins and the RNP complex (Lambs and Parks 2013; Maclachlan and Dubovi 2016). The F protein promotes fusion of the viral envelope with the plasma membrane of the host cell (Maclachlan and Dubovi 2016). The HN protein plays a role in attachment of the virion to the host cell receptor, detachment of the virion from the cell and in tissue tropism independent of the amino acid sequence of the F protein (Huang et al. 2004).

The AOaV-1 replication cycle, represented in figure 3, starts with the attachment of the virion to the host cell receptor, through the binding of HN glycoprotein to host cell sialic acid (SA) receptors. The attachment triggers the F protein promotes fusion of the viral envelope with the plasma membrane of the host cell through a pH-independent mechanism, similar to

other paramyxoviruses (Huang et al. 2004; Bissonnette et al. 2009). After entry, the viral nucleocapsid dissociates from the M protein and is released into the cytoplasm. Then, the viral nucleocapsid is associated with the polymerase complex composed of P and L proteins, to form the ribonucleoprotein complex, and the transcription of the genomic RNA to produce mRNAs is initiated. Newly formed positive-sense RNA intermediates serve as mRNA and use the host cell translation tools to translate viral proteins (Hines and Miller 2012; Lambs and Parks 2013). When sufficient concentration of viral proteins is reached, genome replication takes place. Viral proteins are transported to the cell membrane for the assembly of newly formed virions, mediated by the M protein. The host cell membrane itself becomes modified to form the new viral envelope. New virions are released by budding through the host cell membrane. The neuraminidase activity of the HN protein mediates the detachment of the virion from the cell and removes the sialic acid from progeny virions to prevent self-aggregation (Hines and Miller 2012; Maclachlan and Dubovi 2016).

Figure 3- Representation of AOaV-1 life cycle. Source: original, based on Dortmans 2011.



When the F protein is translated, three identical polypeptide chains assemble into homotrimers that are biologically inactive and host proteases need to cleave the precursor protein, F0, to functional F2 and F1 polypeptides, thus conferring infectivity to progeny virus. Cleaved peptides remain proximal by virtue of linking disulphide bonds. But this cleavage process is not only essential for virus infectivity, is also important to determine de virulence of certain strains. AOaV-1 can be divided into two groups: avirulent strains, that have a single basic amino acid at the cleavage site, and virulent strains, that have multiple basic amino acids

(arginine or lysine) surrounding the glutamine at position 114 and a phenylalanine at position 117 on the cleavage site (de Leeuw et al. 2003; Miller et al. 2010; Hines and Miller 2012). Multiple basic amino acids at the cleavage site make it possible to be cleaved by furin, a host ubiquitously expressed enzyme, and endopeptidases present in the trans-Golgi network, thus facilitating the production of highly infectious virions. On the other hand, avirulent strains have a single basic amino acid at the cleavage site, being recognised only by extracellular proteases with appropriate substrate specificity or by trypsin-like enzymes, mainly present in epithelial cells of the respiratory and gastrointestinal tract. Thus, infectivity of avirulent viruses is restricted to fewer cells and its pathogenic potential is greatly reduced (Hines and Miller 2012; Maclachlan and Dubovi 2016).

2.4.2. Genetic classification

Given the clinical and economical relevance of AOaV-1 to the poultry industry, sequencing and phylogenetic analysis have quickly become the methods of choice for characterization of AOaV-1 strains (Dimitrov et al. 2019). Because of that, several classification systems have been proposed for strain identification and differentiation. However, these systems were created based on different approaches and lacked objective criteria for strain differentiation. When one classification system that used objective criteria was proposed in 2012, the broad circulation of AOaV-1 in poultry and its constant evolution, led to identical naming and the fact that new genotypes were created without applying all of the proposed criteria dictated that a new system would be needed (Diel et al. 2012a; Dimitrov et al. 2019). To address this problem, an international consortium of experts was convened to undertake analysis of AOaV-1 genetic diversity, which generated curated, up-to-date, complete fusion gene class I and class II datasets of all known AOaV-1 for public use (Dimitrov et al. 2019). The new system maintains the former two AOaV-1 classes and existing genotypes, identifies three new class II genotypes, and reduces the number of subgenotypes. A brief review of it is described here.

Class I is composed of a single genotype and three subgenotypes. The evolutionary diversity within the class is low and most isolates come from wild birds. All sequences except one have low virulence (Dimitrov et al. 2019).

Class II is more diverse, contains avirulent and virulent viruses, and is composed of at least 20 distinct genotypes, from I to XXI (genotype XV contains only recombinant sequences, being excluded from the classification system by Dimitrov et al. 2019). Genotypes that are confirmed to be divided into subgenotypes are: I (divided into 4 subgenotypes), V (divided into 2 subgenotypes), VI (divided into 7 subgenotypes), VII (divided into 3 subgenotypes), XII (divided into 2 subgenotypes), XIII (divided into 4 subgenotypes), XIV (divided into 2

subgenotypes), and XVIII (divided into 2 subgenotypes) (Diel et al. 2012a; Dimitrov et al. 2019). Genotypes I, II, III, IV, and IX emerged between the 1930s and the 1960s and are considered ‘‘early” genotypes (Czeglédi et al. 2006). Genotypes V, VI, VII, VIII and XI emerged after 1960 and are considered ‘‘late’’ genotypes (Czeglédi et al. 2006; Diel et al. 2012a). Genotype I comprise low virulence strains from all around the world and some virulent strains from Australia (Gould et al. 2001; Bello et al. 2018). It also comprises well known chicken/Australia/QV4/1966 and chicken/N.Ireland/Ulster/1967 vaccine strains (Bello et al. 2018). Genotype II comprises mainly low virulence strains from America, Africa, Asia and Europe and the LaSota and B1 strains that have been used as live and inactivated vaccines for more than 40 years (Diel et al. 2012a; Bello et al. 2012; Dimitrov et al. 2016). Genotype III strains were mostly isolated before 1960 in Japan, while a few others were isolated from Taiwan in 1969 and 1985, from Pakistan in 1974, from Zimbabwe in 1990 and China in the 2000s (Yu et al. 2001; Czeglédi et al. 2003; Miller et al. 2010; Dimitrov et al. 2016; Bello et al. 2018). All viruses of this genotype are virulent based on the predicted amino acid sequence at the cleavage site of the fusion protein (Dimitrov et al. 2016). Genotype IV viruses were the predominant viruses isolated in Europe before 1970 and include the extensively characterized Herts/33 strain (Miller et al. 2010; Dimitrov et al. 2016). Nowadays, it is possible that strains from these genotypes are no longer maintained in poultry, given the lack of report of contemporary isolates in genetic repositories (Dimitrov et al. 2016). Genotype V emerged in 1970s and is known for being frequently isolated in South Central America from poultry species (Perozo et al. 2008; Rue et al. 2010; Diel et al. 2012a). Genotype VI is the most diverse among all AOaV-1 genotypes and, unlike other AOaV-1 genotypes, it has been isolated in all continents, except Antarctica (Dimitrov et al. 2016b). Many strains that belong to it have been a part of the panzootic in Columbiformes and many of them are known as PPMV-1, an antigenic variant of AOaV-1 that can be distinguished from other AOaV-1 through haemagglutination inhibition test (HI) using specific monoclonal antibodies (mAb) (Aldous et al. 2004; Dimitrov et al. 2019). They appear to be extremely well adapted to some Columbiform species, and only rarely infect chickens *(Gallus gallus domesticus)* (Aldous et al. 2014; Dimitrov et al. 2019). Genotype VII is known for being associated with AOaV-1 outbreaks in poultry from the Middle East and Asia. These strains have been spreading to other hosts, such as geese, and to other locations in the world, while also increasing in virulence (Liu et al. 2003; Huang et al. 2004; Miller et al. 2010; Perozo et al. 2012). Genotype VIII isolates have been reported in chickens from Argentina, China and Malaysia between 1960 and 1980, as well as South Africa and Singapore early in the 1960’s (Herczeg et al. 1999; Abolnik et al. 2004; Murulitharan et al. 2013). However, they have not been reported recently, so it is possible that they are no longer circulating in poultry (Dimitrov et al. 2016). Genotype IX strains are all considered virulent and their most ancestral isolate was recovered from China in the 1940s

(Qiu et al. 2011). Most isolates from this genotype are from poultry from China between 1985 and 2011 (Liu et al. 2003; Qiu et al. 2011; Xie et al. 2013). Genotype X contains low virulence viruses that have been isolated from waterfowl and shorebirds in North America between the 1980s and the 2000s (Kim et al. 2007; Diel et al. 2012a). Genotype XI comprises isolates from poultry from Madagascar isolated between 2008 and 2011 (Maminiaina et al. 2010; Dimitrov et al. 2019). Genotype XII comprises virulent isolates from poultry in South America and from geese in China (Diel et al. 2012a; Diel et al. 2012b). Genotype XIII contains virulent viruses isolated in Russia, Iran and Pakistan between 1995 and 2008 (Diel et al. 2012). Genotype XIV comprises virulent viruses isolated in West and Central Africa since 2006 and are thought to be relatively recent (Diel et al. 2012a; de Almeida et al. 2013). Genotype XVI comprises isolates from the Dominican Republic from 1986 and 2008 and a Mexican isolate from 1947 (Courtney et al. 2013; Dimitrov et al. 2019). Genotypes XVII and XVIII comprise virulent strains isolated from West and Central Africa between 2006 and 2017, probably still occurring until this day (Diel et al. 2012a; Bello et al. 2018; da Silva et al. 2020). Genotype XIX strains are mainly associated with outbreaks in double-crested cormorants (Phalacrocorax auritus) in North America (Rue et al. 2010; Diel et al. 2012c). Genotype XX has some of the oldest available AOaV-1 isolates, that were previously identified as members of genotype VI. All of these were isolated from chickens (Dimitrov et al. 2019). Genotype XXI contains viruses isolated from chickens and Columbiformes in Europe, Africa and Asia between 2005 and 2016 (Snoeck et al. 2013; Van Borm et al. 2012; Wajid et al. 2016; Sabra et al. 2017; Dimitrov et al. 2019). Genotype XXI strains were also previously assigned to genotype VI. At the root of genotype XXI, is a clade of chicken isolates from Ethiopia collected between 2011 and 2012, that have previously been assumed to form a different subgenotype of genotype VI (de Almeida et al. 2013; Dimitrov et al. 2019).

2.4.3. Newcastle disease definition according to World Organization for Animal Health (OIE)

Newcastle disease (ND) is a notifiable disease in Portugal, according to Decree-law no 39 209, of 14 of May 1953. It is a notifiable disease to the European commission according to Directive 82/894/CEE and figures in the list of notifiable diseases of the OIE.

In legal terms, it is very important to have a clear and defined definition of AOaV-1 to create a reasonable and effective legal framework of control measures. According to OIE, ND is defined as an infection of poultry caused by AOaV-1 that meets one of the following criteria for virulence: a) the virus has an intracerebral pathogenicity index (ICPI) in day-old chicks ≥0.7 or the presence of multiple basic amino acids (at least three arginine or lysine) has been demonstrated at the C-terminus of the F2 protein, between residues 113 and 116 and presence of a phenylalanine at residue 117, which is the N-terminus of the F1 protein (OIE 2018a).

Since ND has a legal framework and AOaV-1 infection is only designated as ND when infection of poultry occurs. In this work we use the term “AOaV-1 infection” when talking about wild animals.

2.4.4. Hosts

AOaV-1 has been reported in at least 241 bird species worldwide and from numerous bird orders but may have the potential to infect most, if not all, of bird species (Maclachlan and Dubovi 2016). Chickens are considered the most susceptible species, while aquatic birds are among the least susceptible, normally carrying the virus asymptomatically (Alexander 2000).

AOaV-1 can also infect humans being considered by most authors as a minor zoonotic disease (Kuiken et al. 2018). It has not been isolated from naturally infected non-human and non-avian hosts except for one report of isolation in one calf in 1952 and another report of isolation in two sheep in 2012 (Yates et al. 1952; Sharma et al. 2012).

2.4.5. Transmission

The main routes of transmission are through ingestion or inhalation of faecal contaminated material (Hines and Miller 2012; Maclachlan and Dubovi 2016). Transmission can also occur through contact of infected material with mucous membranes or through contact of infected material with the eggshell after the egg has been laid (Hines and Miller 2012).

Humans can harbour the virus in the conjunctival sac and disseminate it (Hines and Miller 2012).

2.4.6. Clinical outcomes of avian infections

Clinical signs of different strains vary greatly depending on the host species. Among poultry, chickens normally present the more severe clinical signs (Hines and Miller 2012). Waterfowl and other aquatic birds can be infected with AOaV-1 virulent to chickens and show little, if any, clinical signs (Hines and Miller 2012).

Some authors classify ND in chickens into three different pathotypes: lentogenic, mesogenic and velogenic (Hines and Miller 2012). Lentogenic ND can range from asymptomatic enteric, when replication is limited to the gastrointestinal tract and there are no respiratory signs, to mild respiratory and gastrointestinal signs in adult chickens. Young susceptible birds can develop more severe respiratory disease which may lead to increased susceptibility to secondary infections and death. The mesogenic pathotype has intermediate virulence, occurring systemic infection, respiratory disease and drops in egg production in most cases. Rarely, neurological signs can also occur. Velogenic viruses have high virulence and cause systemic disease that often leads to death. They can be divided into velogenic viscerotropic ND (vvND) and velogenic neurotropic ND (vnND). vvND causes acute infection

of the gastrointestinal mucosa leading to haemorrhagic lesions and death (Hines and Miller 2012). Before death, other clinical signs such as prostration, diarrhoea, muscular tremors, paralysis, and oedema around the eye may occur. vnND infection normally leads to respiratory distress followed by neurological disease. Morbidity of both vvND and vnND is around 100%, with vnND having a lower mortality, of around 50% in adult birds (Hines and Miller 2012). In chickens, the incubation period varies from two to fifteen days (Hines and Miller 2012).

Reports regarding clinical signs in birds are scarce and are probably different between species. In *P. auritus*, *Pelecanus erythrorhynchos* and *Larus spp*. paresis or paralysis of limbs, head and/or neck, inability to hold affected wing tucked against e body, torticollis, head or body tremors, ataxia and blindness have been reported (Wobeser et al. 1993; Kuiken et al. 1998; Glaser et al. 1999). In common pigeons (*Columba livia*) infected with velogenic AOaV-1, classic signs such as digestive signs (watery or haemorrhagic diarrhoea), respiratory signs (rhinitis, dyspnoea, and congestion of pharyngeal and laryngeal mucous membranes) and neurological signs (torticollis, ataxia and neck tremor) occur (Vindevogel et al. 1972; Marlier and Vindevogel 2006). Gross lesions observed at necropsy are like those seen in chickens. Morbidity can reach 70% and mortality 40% (Vindevogel et al. 1972; Marlier and Vindevogel 2006). In Passeriformes, a study reported depression and weakness of the limbs in all five species infected as well as diarrhoea, incoordination, and paralysis of various degrees in four of the five species (Khalafalla et al. 1990).

2.4.7. Clinical outcomes of human infections

Infection with AOaV-1 in humans is uncommon and normally only causes transient conjunctivitis. However, two cases of fatal pneumonia in immunocompromised human patients have been described (Goebel et al. 2007; Kuiken et al. 2018).

2.4.8. Epidemiology

The first known panzootic episode of NDV occurred in 1926, in Java, Indonesia and then in Newcastle-upon-Tyne, England in 1927, from where the disease received its name (Alexander et al. 2012). Although the disease initially spread rapidly in Asia, it took between 16 to 40 years to become a true panzootic (Alexander et al. 2012). In the late 1960s the second panzootic emerged and its worldwide spread took only four years, with uncountable economic losses (Alexander et al. 2012).

AOaV-1 is considered today one of the most important diseases in poultry (Kapczynski et al. 2013; Cheng et al. 2016). Furthermore, the fact that it is endemic to various countries in the world composes a constant threat to worldwide poultry production and, especially in developing countries of Asia and Africa where poultry is one of the main food sources it poses an important threat to food safety (Miller et al. 2010; Cappelle et al. 2015). Currently, sporadic

outbreaks of NDV occur across the world, especially in European Union (EU) countries (Miller et al. 2010; Alexander et al. 2012).

In wild birds, most cases document the occurrence of low virulence AOaV-1 infections, with few cases documenting infections with virulent strains (Alexander et al. 2011). Although, there are three described exceptions to the apparent absence of endemic virulent AOaV-1 in wild birds: the panzootic in pigeons, its presence in *P. auritus* in North America and the circumstantial evidence that there may exist a spread of AOaV-1 wild bird of Europe (Alexander et al. 2012).

The first description of virulent AOaV-1 infection in domestic pigeons occurred in the late 1970s in the Middle East and in the 1980s the virus had also affected pigeons in Europe (Biancifiori and Fioroni 1983; Kaleta et al. 1985). After that, it spread quickly to all parts of the world, probably due to pigeon races, bird shows and international trades, with virulent AOaV-1 being isolated in wild Columbiformes in the EU on various occasions, mainly from Eurasian collared doves (*Streptopelia decaocto*) and European turtle doves (*Streptopelia turtur*) (Alexander 2011). Some authors suggest that, like in pigeons, AOaV-1 has become enzootic in these species in some parts of Europe. One study of isolates from outbreaks in Italy between 2000 and 2001 demonstrated that these isolates were genetically distinct from pigeons contemporaneous isolates although it is unclear whether this applies to isolates from *S. decaocto* and *S. turtur* from other countries (Terregino et al. 2003).

In *P. auritus*, outbreaks of AOaV-1 were first described in 1990 in Canada (Wobeser et al. 1993). In the following years it spread across North America and has since killed thousands of birds (Kuiken et al. 1998; Allison et al. 2005; MNBAH 2019).

In Europe, there is some circumstantial evidence that there may exist a spread of AOaV-1 due to wild birds, since there are some reported cases where there is a strong evidence that virulent viruses in poultry have derived through mutation from low virulence viruses in wild birds (Alexander 2001). For example, in 1997 AOav-1 outbreaks occurred in 11 poultry populations in Great Britain, with these isolates revealing a close similarity to isolates from poultry outbreaks in Scandinavian countries in 1996 and an isolate from a feral goosander (*Mergus merganser*) collected in Finland in 1996 (Alexander et al. 1999). Unusual migratory movements were made by wild birds at the end of 1996 and beginning of 1997, suggesting that these birds may have spread the virus. After a few years, in 2001, a closely related virus was obtained from a *Phalacrocorax sp.* in Denmark and, between 2002 and 2004, in poultry in Finland, Denmark and Sweden and, in 2005, in an outbreak in pheasants in Great Britain (Aldous et al. 2007; Alexander 2011). Although, these findings provide circumstantial evidence

of virus transference between wild and domestic birds populations, the role of AOaV-1 in wild birds and its epidemiology remain largely unknown.

In Portugal, the first detection and isolation of AOaV-1 was in 1947 from chicken cadavers (Henriques et al. 2017). The disease then disseminated to the whole country, reaching the highest prevalence in the 1990s, and a gradual decrease in the number of cases in the 2000s (Henriques et al. 2017). In the last six years, only 2 reports of AOaV-1 have been described in Portugal. The first one occurred in Porto Santo, Madeira in July 2015 and was associated with a mortality event in both pigeons and *S. turtur*. The second occurred in July 2017 in two racing pigeons from two different farms in Mira (OIE 2020).

2.4.9. Diagnosis

Clinical signs of AOaV-1 are relatively nonspecific, so diagnosis must be confirmed by viral isolation, serology, viral detection by RT-PCR or RT-qPCR or immunohistochemical staining assays (Hines and Miller 2012; Maclachlan and Dubovi 2016).

Haemagglutination-inhibition (HI) serological assays and RT-PCR assays with subsequent sequence analysis are normally made (Hines and Miller 2012; Maclachlan and Dubovi 2016). Although, one must know the bird's vaccination history to interpret serological and molecular diagnostic methods results, due to the interference of live-attenuated virus vaccines (Hines and Miller 2012; Maclachlan and Dubovi 2016).

Viral isolation can be attempted from tracheal/oropharyngeal swabs or cloacal swabs sampled from live and dead birds or tissues from dead birds. In some cases, culture systems such as cell culture or allantoic sac inoculation of 9-10 day old specific-pathogen-free embryonated eggs can be used (Hines and Miller 2012; Maclachlan and Dubovi 2016).

2.5. Influenza A virus (IAV)

2.5.1. Characterization – viral genome, structure, and life cycle

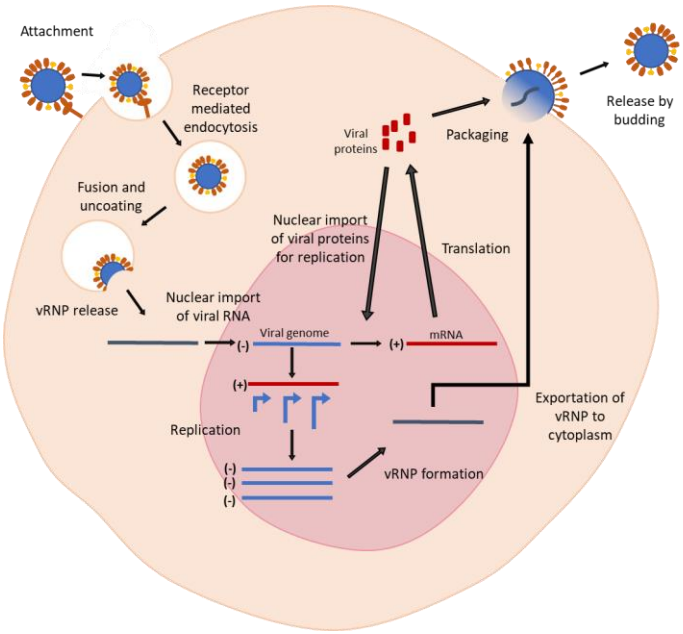
Influenza A virus (IAV) belongs to genus *Alphainfluenzavirus* of *Orthomyxoviridae* family (Samji 2009; Lambs and Parks 2013; Nuñez and Ross 2019). Influenza A is a negative-sense, single-stranded RNA and virions are normally pleomorphic, but filamented and spherical forms with 80-120 ηm of diameter also occur (Palese & Shaw, 2013). Virions are enveloped with large glycoprotein spikes surrounding eight symmetrical nucleocapsid segments. The Influenza A genome includes eight segments that encode for at least 10 viral genes namely: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NS1), non-structural protein 2 (NS2; also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1) and polymerase basic protein 2 (PB2) (Samji 2009; Lambs and Parks 2013; Nuñez and

Ross 2019). In some IAV strains, accessory proteins, such as PB1-F2 and PB1 N40 can also be coded. The NA, HA and M2 proteins are embedded in the envelope lipid bilayer derived from the host cell, being HA the most abundant envelope protein. HA protein is necessary for attachment of the virion to the host cell while NA is essential for viral budding. The M1 protein, that underlies the envelope, forms a matrix holding the viral ribonucleoproteins (vRNP). The vRNP are composed of the viral RNA wrapped around the NP and NS2 proteins. M2, NS1 and NS2 are essential for viral replication The PB1, PB2 and PA proteins make up the polymerase complex, responsible for viral transcription and replication (Lambs and Parks 2013; Maclachlan and Dubovi 2016; Nuñez and Ross 2019).

IAV replication cycle, represented in figure 4, begins with the entrance of the virion into the cell via receptor-mediated endocytosis. Then, the low pH environment of the endosome induces not only a conformation change in HA0, allowing the fusion of the viral and endosomal membrane, but it also opens M2 ion channel (Holsinger and Lamb 1991). This ion channel acidifies the viral core, ultimately leading to dissociation of viral M1 matrix protein from vRNPs and releasing of vRNPs into cytoplasm. Unlike most RNA viruses, influenza viruses replicate in the host cell nucleus, so these vRNPs are actively transported into the nucleus to start transcription and genome replication. Transcription of the negative-stranded RNAs into either mRNAs, which directs viral protein synthesis, or positive-strand RNA, which is used as a template for the viral RNA genome synthesis; the newly synthesized viral genome segments and proteins are assembled to form new vRNPs in the nucleus (Samji 2009; Lambs and Parks 2013; Nuñez and Ross 2019). Then, these vRNPs are exported from the nucleus and are assembled in the cytoplasm for final assembly. Virions are formed by budding, incorporating both M1 protein and nucleocapsids that align below patches on the plasma membrane in which HA, NA and M2 proteins have been inserted (Lambs and Parks 2013). Finally, and to allow the virions to be released, the NA spikes cleaves the terminal SA residues on the plasma membrane that would otherwise hold the virions on the cell surface (Maclachlan and Dubovi 2016; Nuñez and Ross 2019).

According to some authors, the main factor which influences host susceptibility to infection is the receptor conformation of host cells. While human influenza viruses bind preferably to SA-α2,6-Gal-terminated saccharides, which are present in human epithelial cells, avian influenza viruses bind preferably to SA-α2,3-Gal-terminated saccharides, which are more prominent on avian cells. This difference is supposed to be one of the factors that lessens the risk of crossing the species barrier (Alexander 2007).

Figure 4 - Representation of IAV replication cycle. Source: original, based on Willey et al. 2008.



2.5.2. IAV characterization and genetic evolution

IAVs of birds can be divided in low pathogenic avian influenza viruses (LPAIV) and highly pathogenic avian influenza viruses (HPAIV), depending on the amino acid motif present at the cleavage site of the HA molecule (Nuñez and Ross 2019). The HA glycoprotein is produced as a precursor, HA0, that requires posttranslational cleavage by host proteases to be functional allowing viruses to become infectious (Rott 1992). LPAIV contain only one basic amino acid at the cleavage site and needs to be cleaved by host trypsin-like enzymes and, thus, can only replicate where these enzymes are found, such as the respiratory and intestinal cells. HPAIV, on the other hand, contain multiple basic amino acids (i.e. arginine and lysine) at their cleavage site and are probably cleaved by a ubiquitous furin cellular protease, allowing the viruses to replicate throughout most tissues (Nuñez and Ross 2019).

Influenza viruses tend to suffer mutations that influence the virulence and viral evolution with high frequency (Proença-Módena 2007). During replication of influenza virus, antigenic changes sometimes occur. Minor changes, called antigenic drifts, occur by accumulation of point mutation during transcription of the viral genes, due to the lack of a proofreading mechanism in the viral RNA polymerase. These changes, especially those that occur in genes which code the HA and NA, can generate new strains of the influenza virus and cause new outbreaks, since there is no significant pre-existing protective immunity in the population (Al Faress et al. 2005). More significant changes, called antigenic shift, occur when genetic reassortment happens between two viral strains that infect the host simultaneously, resulting in the acquisition of new gene segments. This can result in the emergence of new viral subtypes, which may have increased virulence (Ghedin et al. 2005).

Influenza A viruses are classified based on the antigenic properties of the glycoproteins haemagglutinin (HA) and neuraminidase (NA) into 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11) (Nuñez and Ross 2019). Prior to 2012, only 16 HA subtypes and nine NA subtypes were described, circulating primarily in avian reservoirs. Although, during 2012, 2013 and, more recently, 2018, newly H17 and H18 and N10 and N11 were described in bats (Tong et al. 2012; Tong et al. 2013; Campos et al. 2019). Until this day, these HA and NA subtypes continue to be reported only in bat species (Campos et al. 2019).

2.5.3. Avian influenza definition according to OIE

The definition as found in OIE Terrestrial Animal Health Code is that avian influenza is a notifiable disease characterized by being an avian infection caused by any HPAIV or by H5 and H7 LPAIV. All the other LPAI subtypes are non-notifiable (OIE 2018b).

To avoid confusion with the scientific use of “avian influenza”, HPAIV, H5/H7 LPAIV and IAV will be used instead. The latter indicates any H1–H16 influenza virus from birds.

2.5.4. Hosts

IAV affects mainly waterfowl of the order *Anseriformes* and *Charadriiformes* (Alexander 2000; Olsen et al. 2006; Venkatesh et al. 2018). Species of these orders are known to harbour a wide range of LPAIVs (Alexander 2000; Alexander 2007). Although, IAV can also affect other species of wild and domestic birds and even humans (Alexander 2000). It has been detected in a wide variety of animal species including pigs, horses, seals, whales, ferrets, tigers, dogs and cats (Cauldwell et al. 2014; Kuchipudi and Nissly 2018).

Subtypes H17N10 and H18N11 have only been described in bats, and it is still unknown if they have any capability to naturally infect other mammal and avian species. Although, contrary to other IAV viruses, they use MHC-II as a cell entry mediator, which is a highly conserved entry found in many vertebrates, so its potential to infect humans and other species cannot be excluded (Karakus et al. 2019). Considering this, it is possible that IAV’s host range could be much broader than currently known (Kuchipudi and Nissly 2018).

2.5.5. Clinical outcomes of avian infections

In poultry LPAIV normally cause a decrease in egg laying, anorexia, lethargy, respiratory disease, and sinusitis. Although, they can also cause serious disease in immunocompromised individuals (Maclachlan and Dubovi 2016). Infected wild waterfowl normally does not show clinical signs (Maclachlan and Dubovi 2016).

In poultry, HPAIV normally causes sudden death. When birds survive for more than 48 hours, there is a decrease in egg laying, respiratory distress, sinusitis, diarrhoea, oedema of the head, face and neck and cyanosis of unfeathered skin. Birds that survive for more than 3-

5 days may show neurological signs such as tremors of the head and neck, torticollis, inability to stand and abnormal postures (Maclachlan and Dubovi 2016).

2.5.6. Clinical outcomes of human infections

Human symptoms of IAV infections range from mild such as conjunctivitis, flu-like symptoms (fever, cough, myalgia) sometimes accompanied by abdominal pain, diarrhoea and vomiting, to severe respiratory illness, with symptoms such as shortness of breath, pneumonia, acute respiratory distress, and respiratory failure, and/or neurologic symptoms, such as altered mental status and seizures. In some cases, the involvement of other organ systems can also occur (Alexander 2008).

2.5.7. Transmission

Transmission can occur through the respiratory tract or faecal/oral tract (Alexander 2008). Bird-to-bird transmission depends on the strain of virus, bird species and environmental factors (Alexander 2008). While most LPAIV are primarily transmitted through the faecal/oral route, several studies demonstrate that H5N1 HPAIV were primally transmitted through the respiratory route. This change was important in the virus epidemiology and in its spread to mammals (Perez et al. 2003; Humberd and Webster 2006).

According to some studies, in both natural and experimental infections, virulent viruses had poorer transmission between chickens and turkeys (*Meleagris gallopavo domesticus*) than viruses of low pathogenicity (Alexander 2008). This is possibly due to HPAIV normally causing rapid death, limiting the amount of virus excreted during such infections (Alexander 2008).

2.5.8. Epidemiology

IAV was first defined in 1878 as fowl plague and for approximately 100 years it only occurred rarely. In 1901 it was shown that the causative agent was a virus, but it was only in 1955 that the scientific community demonstrated an association between this and other viruses isolated from birds with mammalian influenza A viruses (Alexander 2000).

The first isolation of IAV from wild birds was a HPAI H5N3 virus isolated from a common tern (*Sterna hirundo*) in 1961 in South Africa, but only a decade later was further investigation of IAV in wild birds undertaken. In 1972, a LPAIV was first isolated from wild birds (Becker et al. 1966; Alexander 2000). Today, more than 100 subtypes of IAVs have been detected in birds (Rejmanek et al. 2015; Philippon et al. 2020). Although, only a few of them were reported to cause human infections and most of them have been assessed to be of low concern due to its low severity in humans or limited cross-species transmissibility. Subtypes of IAV of avian origin reported in humans are H5N1, H5N6, H6N1, H7N2, H7N3, H7N4, H7N7, H7N9, H9N2, H10N7 and H10N8 (Philippon et al. 2020).

In humans, the first know infection with an avian IAV strain was the isolation of H7N7 from a woman who lived in England and kept ducks, in 1996. The only clinical sign presented was conjunctivitis (Kurtz et al. 1996; Banks et al. 1998).

Between 1959 and 1995 there were few documented cases of emergence of HPAIV in poultry, however, in 1996, everything changed. In 1996 a strain of H5N1 emerged in geese in Southern China, namely A/goose/Guangdong/1/1996, and in 1997, human infections with this strain were reported during an outbreak in poultry in Hong Kong. Since 2003, the virus has spread from Asia to Europe and Africa countries, even becoming endemic in poultry populations in some of these countries and it has persisted ever since. It has resulted in infection, and culling of millions of birds, 861 human cases and 455 human deaths, with 160 human cases and 48 deaths occurring between 2015 and 2020 (WHO 2020).

In 2013, human infections with H7N9 virus were reported for the first time in China. Since then, the virus has spread in the poultry population across the country and resulted in over 1500 reported human cases and 616 human deaths. The virus has been reported in chickens, pigeons, ducks, turkeys, peafowl (*Pavo cristatus*), a tree sparrow (*Passer montanus*) and a magpie robin and most positive birds were from live bird markets, vendors and breeding farms (FAO 2019).

2.5.10. Diagnosis

Like AOaV-1, IAV clinical signs are extremely variable and non-specific so diagnosis must be confirmed by viral detection with RT-PCR or RT-qPCR, by viral isolation or by serological tests, such as HI tests or ELISAs (Maclachlan and Dubovi 2016).

Viral isolation can be attempted from tracheal/oropharyngeal swabs or cloacal swabs sampled from live and dead birds or tissues from dead birds. In some cases, culture systems such as cell culture or allantoic sac inoculation of 9-10 day old specific-pathogen-free embryonated eggs can be used (Hines and Miller 2012; Maclachlan and Dubovi 2016).

In serological tests, such as ELISA, an initial screening is performed using a broad serological test for various influenza viruses, followed by 16 different haemagglutinin- and nine neuraminidase-specific tests for serotyping (Maclachlan and Dubovi 2016).

3. MATERIAL AND METHODS

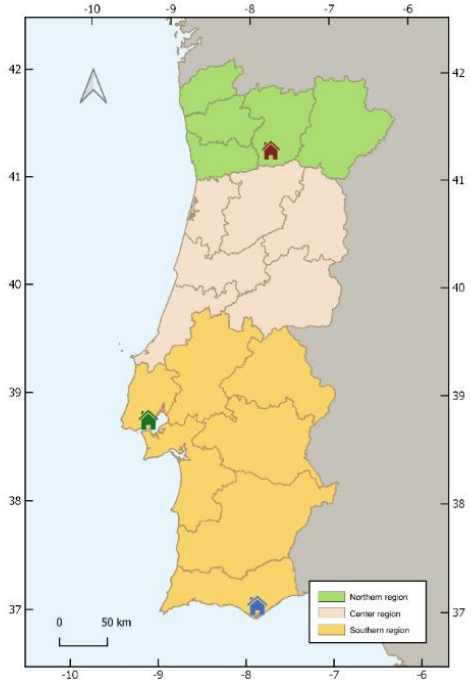
3.1. Sample origin

Samples were collected from birds found dead or hospitalized in three different wildlife rehabilitation centres belonging to the National Network of Rehabilitation of Fauna: the wildlife rehabilitation centre of Lisbon (LxCRAS) (38° 44' 24.50" N 9° 11' 12.68" W), the wildlife rehabilitation and research centre of Ria Formosa natural park, in Algarve (RIAS) (37°02'03.7"N 7°48'47.1"W), and the wildlife rehabilitation centre of the University of Trás-os-Montes e Alto Douro's Veterinary Teaching Hospital (CRAS-HVUTAD), in Vila Real (41º17'22.00''N 7º 44'26.70''W) (figure 5).

A total of 192 birds were sampled, 82 in vivo and 110 *post mortem* (Appendix 3). When possible, blood sample, oropharyngeal and cloacal swabs were collected from live animals; brain, spleen and large intestine samples were collected from dead birds. In some cases, not all samples were collected due to health status of the animal, to unavailability of material and/or staff or due to cadaver decomposition stage.

It is important to note that although the samples were collected during the period of the animals' hospitalization in the wildlife rehabilitation centres, some of them were rescued from other districts of the country and then transported to the centres, either by citizens or by the competent authorities. The animal's place of origin is routinely recorded in the centre's database and it was this location that was used for the posterior analysis in this work.

Figure 5- Localization of the wildlife rehabilitation centres where samples were collected. Red house represents CRAS-HVUTAD, green house represents LxCRAS and blue house represents RIAS. Source: original.



3.2. Sample collection, processing, and storage

Samples from alive animals were collected between 8th March 2018 and 9th June 2019.

Blood was mainly collected from the *vena cutanea ulnaris superficialis*, also known as basilic vein. In some cases, due to the species' anatomical particularities or health status of the animal, blood was collected from the medial metatarsal vein. The blood quantity collected varied between 0.2 mL and 1 mL, depending on weight, species, and health status of the animal. Blood collection was made with 1 mL syringes (Terumo Corporation, Tokyo, Japan) and 25 G (0.5x16 mm) or 23 G (0.6x25mm) needles (Sterican®, B.braun), then placed on an EDTA tube (kima test® K3 EDTA, 0.5 mL, Vacutest Kima, Padova, Italy) and centrifuged at 3000 x *g* for 5 minutes. After centrifugation plasma was collected, transferred to a labelled sterile 1.5 mL microcentrifuge tube, and kept at -20 ºC until further analysis. When possible, oropharyngeal and/or cloacal swabs were also taken and placed on labelled sterile 1.5 mL microcentrifuge tubes.

On cadavers approximately 1 cm^3 of brain, spleen and large intestine were collected and placed in individually labelled sterile 1.5 mL microcentrifuge tubes. Oropharyngeal and/or cloacal swabs were also taken and placed on labelled sterile 1.5 mL microcentrifuge tubes. Afterwards, the collected samples were kept at -20 ºC for a maximum of one month and then transported to the INIAV virology laboratory and kept at -80 ºC until further analysis. An internal label was attributed to each individual sample and was kept unaltered during all work phases.

A total of 127 oropharyngeal swabs, 89 cloacal swabs, 73 blood samples, 98 brain samples, 89 spleen samples and 85 large intestine samples were collected.

3.3. Sample preparation

For each animal, a pool with brain, spleen, large intestine and/or oropharyngeal swab was prepared. Depending on the biological material collected, three different protocols were made.

In animals with only organ samples, approximately 0.2 cm^3 of each organ was cut and homogenised with 300 µL of Phosphate-Buffered Saline (PBS) on a petri dish (Nunclon™, Thermo Fisher Scientific, Waltham, USA).

In animals with only oropharyngeal and cloacal swabs, 500 µl of PBS were added to each individual tube containing the swabs, the tube was vortexed for 15 seconds and was left to stand for at least 10 minutes. Then, 250 µL of each homogenate were collected and mixed together to make the final homogenate, with a total volume of 500 µL.

In animals with only organ samples and one of the swabs, 500 µL of PBS were added to the tube containing the oropharyngeal swab, the tube was then vortexed for 15 seconds and then it was let to stand for at least 10 minutes. Approximately 0.2 cm^3 of each organ was cut and macerated on a petri dish with 300 µL of PBS from the oropharyngeal tube and 200 µL of new PBS to make the final homogenate.

Some animals had organ samples and both swabs (oropharyngeal and cloacal swab). In these cases, 500 µL of PBS were added to the tube containing the oropharyngeal swab and another 500 µL of PBS were added to the tube containing the oropharyngeal swab. Both tubes were vortexed for 15 seconds and left to stand for at least 10 minutes. Then, the organ samples were processed, with approximately 0.2 cm^3 of each organ being cut and macerated on a single petri dish with 200 µL of the homogenate from the oropharyngeal tube, 200 µl of the homogenate from the cloacal tube and 100 µL of PBS to make the final volume of 500 µl.

All the final products were transferred to a labelled sterile 1,5 mL microcentrifuge tube, vortexed for 3 seconds and then were kept at 4 ºC until nucleic acid extraction for a maximum of 24 hours.

3.4. Nucleic acid extraction

Total RNA was extracted from tissue samples using a nucleic acid extraction workstation BioSprint96 (Qiagen, Hilden, Germany) with the MagAttract® 96 cador pathogen kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. The extracted nucleic acids were kept at 4 ºC when further analysis was conducted in a maximum period of seven days or at -80 ºC after that period.

In some cases, extracted nucleic acids were pooled in groups of three or five to expand the capacity of the test kits results, potentially reducing the number of tests needed. Whenever a pool tested positive, work-up of individual samples was initiated.

3.5. Real-time polymerase chain reaction (RT-qPCR) conditions

All the RT-qPCR amplifications were performed using the CFX96™ Optical Reaction Module (Bio-Rad) and One-step NZYSpeedy RT-qPCR Probe kit (NZYTech, Portugal).

For WNV screening the protocol developed by Barros et al. (2013) that targets the NS2A protein gene was used. The reaction volume used was 20 µL containing 0.5 µL of probe 3612/3620 (10 pmol/µL), 0.5 µL of F3548 primer (50 pmol/µL), 0.5 µL of R3683 primer (50 pmol/µL), 10 µL of Mix 2X (NZYSpeedy), 0.8 µL NZYRT mix (NZYSpeedy), 0.7 µL of Rnase-free water and 7 µL of the DNA template. The PCR mixtures were subjected to an amplification protocol including 20 minutes at 50 ºC for reverse transcription, 5 minutes at 95 ºC to activate

the Taq DNA polymerase followed by 50 cycles of denaturation, 20 seconds at 95 ºC, annealing, 30 seconds at 55 ºC and extension, 30 seconds at 72 ºC.

For the positive and negative RT-qPCR controls, RNA from WNV-Eg101 strain and nuclease free water were used, respectively. At the end of each PCR annealing step the amount of fluorescence emitted was measured and the threshold cycle (Ct) value was registered. Ct values >50 were considered negative.

For USUV screening the protocol developed by Cavrini et al. (2011) that targets the NS5 protein gene was used. The reaction volume used was 20 µL containing 0.5 µL of probe (10 pmol/µL), 0.5 µL of forward primer (50 pmol/µL), 0.5 µL of reverse primer (50 pmol/µL), 10 µL of Mix 2X (NZYSpeedy), 0.8 µL NZYRT mix (NZYSpeedy), 0.7 µL of Rnase-free water and 7 µL of the RNA template. The RT-PCR mixtures were subjected to an amplification protocol consisting of 20 minutes at 50 ºC for reverse transcription, 5 minutes at 95 ºC to activate the Taq DNA polymerase then 50 cycles of denaturation, 20 seconds at 95 ºC, annealing, 30 seconds at 55 ºC and extension, 30 seconds at 72 ºC. For the positive control, a known positive sample was used. Nuclease free water was used as a negative control. Ct values >50 were considered negative.

For AOaV-1 screening, the protocol developed by Fuller et al. (2010) that targets a region of the L protein gene was used. The reaction volume used was 20 µL containing 0.5 µL of LProMGB probe (5 pmol/µL), 0.5 µL of LProMGB2 probe (5pmol/µL), 0.25 µL of forward primer (12.5 pmol/µL), 0.25 µL of reverse primer (12.5 pmol/µL), 10 µL of Mix 2X (NZYSpeedy), 0.8 µL NZYRT mix (NZYSpeedy), 0.7 µL of nuclease free water and 7 µL of the RNA template. The PCR mixtures were subjected to amplification cycles consisting of 20 minutes at 50 ºC for reverse transcription, 5 minutes at 95 ºC to activate the Taq DNA polymerase then 50 cycles of denaturation, 10 seconds at 95 ºC, annealing, 30 seconds at 50 ºC and extension, 30 seconds at 70 ºC. For the positive control, a known positive control sample was used. Nuclease free water was used as a negative control. Ct values >40 were considered negative. Ct values ≥37 for two consecutive runs with the same isolate was considered an inconclusive result and, for this work's purpose, considered as a negative result.

For IAV screening the protocol developed by Spackman et al. (2002) that targets the M protein gene was used. The reaction volume used was 20 µL containing 0.5 µL of probe 64 (10 pmol/µL), 0.5 µL of M25 primer (50 pmol/µL), 0.5 µL of M124 primer (50 pmol/µL), 10 µL of Mix 2X (NZYSpeedy), 0.8 µL NZYRT mix (NZYSpeedy), 0.7 µL of nuclease free and 7 µL of the DNA template. The PCR mixtures were subjected to amplification cycles consisting of 20 minutes at 50 ºC for reverse transcription, 5 minutes at 95 ºC to activate the Taq DNA polymerase then 50 cycles of denaturation: 15 seconds at 95 ºC, annealing and extension, 25

seconds at 60 ºC. For the positive control, a known control positive sample was used. Nuclease free water was used as a negative control. Ct values >50 were considered negative.

3.6. Conventional RT-PCR conditions

For conventional RT-PCR conditions AgPath-ID One Step RT-PCR kit Thermo Fisher Scientific) was used.

While some samples were screened for WNV and USUV by specific RT-qPCR protocols described above, others were screened by an in-house Pan-flavivirus PCR protocol that targets the NS3 protein gene to be more cost effective. For Pan-flavivirus screening the reaction volume used was 25 µL containing 0.5 µL of 5461F primer (5´- ATGGATGARGCTCAYTTCAC-3´) (50 pmol/µL), 0.5 µL of 5669R primer (5´-GTKATCCATYCRTATCCA-3´) (50 pmol/µL), 12.5 µL of 2X RT-PCR Buffer, 1 µL of 25X RT-PCR Enzyme Mix, 0.5 µL nuclease free water and 10 µL of the DNA template. The amplification cycles included: 20 minutes at 45 ºC for reverse transcription; 10 minutes at 95 ºC to activate the Taq polymerase, 50 cycles of denaturation: 15 seconds at 95 ºC; annealing, 25 seconds at 50 ºC and extension, 30 seconds at 72 ºC and a final elongation step of 7 minutes at 72 ºC. For the positive control, a known positive control sample was used. Nuclease free water was used as a negative control.

When a positive sample was detected in AOaV-1 RT-qPCR screening, a conventional RT-PCR that targets the F protein gene was performed to allow amplicon sequencing (Oberdörfer & Werner,1998). The final reaction volume was 25 µL, containing 0.5 µL of forward primer (50pmol/µL), 0.5 µL of reverse primer (50 pmol/µL), 10 µL of Mix 2X (NZYSpeedy), 4 µL of nuclease free water and 10 µL of the DNA template. The amplification cycles included: 20 minutes at 45 ºC for reverse transcription, 10 minutes at 95 ºC to activate the Taq polymerase followed by 50 cycles of denaturation: 15 seconds at 95 ºC, annealing, 25 seconds at 55 ºC and extension, 30 seconds at 72 ºC and then a final elongation step of 7 minutes 72 ºC. For the positive control, a known positive control sample was used. Nuclease free water was used as a negative control.

The final products of the RT-PCR were run in a horizontal electrophoresis gel. The gel was prepared using TBE (Tris-Borate-EDTA) buffer, 1.5% (w/v) agarose and 10 µL/100 mL of gelRED™ (Biotium, CA, USA). The electrophoretic migration of the products was made at 120 V for 40-60 minutes. To estimate the fragment size, a molecular weight marker Quick Load 100 bp DNA Ladder (New England BioLabs Inc. MA, USA) was included.

The amplicons were observed under ultraviolet light in a Chromato-Vue Transilluminator and compared with the 100 bp marker. The bands with the expected size of

362 bp were excised from the agarose gel using a razor blade and transferred into a microcentrifuge tube to be purified.

3.7. Fragment purification

Amplicons purification was performed at room temperature with the NZYGelpure kit (NZYTech). The excised gel fragment was weighed and 300 µl of binding buffer per 100 mg of gel fragment were added and incubated at 56 ºC until completely melted (between 5-10 minutes). After that, if the solution had turned orange or violet instead of yellow, 10 µl of 3M sodium acetate pH 5.0 was added. Since the fragments had less than 500 bp, a gel volume of isopropanol was added to the solution. The solution was then put in a NZYTech spin column inserted in a collection tube (2 mL) and centrifuged at 13.000 x *g* for 1 minute. Then 500 µL of wash buffer were added and centrifuged for 1 min. The flow-through from the collection tube was discarded and an additional 600 µL of wash buffer were added and centrifuged again for 1 minute to ensure proper washing. Then, the flow-through from the collection tube was discarded and the column was centrifuged for 1 min to remove the residual ethanol. The column was transferred to a 1.5 mL microcentrifuge tube and 50 µL of elution buffer was added to the centre of the column and incubated for 1 min at room temperature. Finally, it was centrifuged once more for 1 min for DNA elution.

3.8. Sequencing analysis (Sanger method)

Sequencing was performed with the ABI Prism BigDye® Terminator Cycle sequencing kit (Applied Biosystems, Foster City, USA). To perform the cycle sequencing the following components were mixed: 2 µL of BigDye® Terminator Ready Reaction Mix, 0.2 µL of primer, 1 µL of BigDye® terminator 5X sequencing buffer, 4 µL of RNA template and 2.8 µL of nuclease free water. Forward primer and Reverse primer were used singly, which meant that for each amplification product at least two sequencing reactions were made. The amplification protocol consisted of 1 minute at 96 ºC for denaturation and 25 cycles of denaturation (10 second at 96 ºC), hybridization (5 seconds at 55 ºC) and elongation (1 minute at 60 ºC).

The sequencing reaction was then purified using sodium acetate and ethanol precipitation. To accomplish that, 1 µL of 125 mM EDTA, 1 µL of 3M NaAc and 25 µL of ethanol (EtOH) 95% were added to each sequence reaction and incubated for 15 minutes at room temperature. The tubes were centrifuged for 20 minutes at 21130 *x g* (at 4 ºC). The formed supernatant was discarded, 85 µL of EtOH 70% were added and the tube was vortexed. After discarding the supernatant, the DNA pellet was washed with 85 µL of EtOH 70%, the tube was vortexed and centrifuged again for 10 minutes at 21130 *x g*. The supernatant was careful discarded, and the pellet was dried. Finally, 20 µL of formamide were added and the tube was vortexed to dissolve the pellet. The nucleotide sequences of the amplicons were determined

on an automated 3500 Genetic Analyzer system (Applied Biosystems) and assembled with MEGA X (Kumar et al. 2018).

3.9. Viral isolation in embryonated chicken eggs

RT-qPCR positive samples AOaV-1 were subjected to viral isolation in embryonated chicken eggs and in cell culture in order to obtain high viral titre which would allow a more thorough downstream characterization by conventional RT-PCR based techniques or by the haemagglutination inhibition test and full genome characterization in future works.

Viral isolation in specific-pathogen-free embryonated chicken eggs was attempted according to OIE and to European standards (OIE 2018a, EC 94/2005). Briefly, 0.4 mL of clarified organ homogenate (centrifuged at 1500 x *g* for 10 minutes at 22 ˚C) and 1.5 mL of PBS buffer were mixed in a 2 mL microcentrifuge tube. Then 2 µL of filtered gentamicin at 50 mg/mL and 20 µL of Gibco Antibiotic-Antimycotic 100x (Thermo Fisher Scientific), containing 10.000 units/mL of penicillin, 10.000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B were added and left to rest for at least 30 minutes at room temperature or 2 hours at 4 ºC. When the supernatant was ready for inoculation, five eggs were prepared. Embryonated eggs with 9 to 11 days old were candled to see the embryo viability. The air chamber limit and the inoculation site were identified with a pencil and the eggs were identified with the inoculation date, the passage number, and the type of sample. The inoculation site was disinfected with alcohol 70% and a small hole was made with a manual device. Then, 0.2 ml (if first passage) or 0.1 ml (if second or third passages) of clarified supernatant obtained from the oropharyngeal/tracheal and cloacal swabs or from the organ homogenate was inoculated into the allantoic sac of each egg with a 25 G needle and a 1 ml syringe. All eggs were sealed with paraffin and incubated at 37 ºC with 62% humidity for seven days. The eggs were viewed using a candling lamp three times per day to check for embryo vitality and the number of dead embryos was recorded daily. After seven days, eggs with live embryos were refrigerated at 4 °C for at least 6 h to cause embryo death. Eggs were then open in a laminar flow cabinet and the allantoic fluid was harvested to test for the presence of HA activity. The mortality of embryonated eggs in the first 24 hours post-inoculation was considered non-specific and related to the inoculation technique. The procedure was repeated until the third blind passage. Then, allantoic liquid was collected and submitted to rapid hemagglutination inhibition test and RT-qPCR for AOaV-1, as previously described, if considered pertinent (Fuller et al. 2010).

3.10. Viral isolation in cell culture

Since some strains of AOaV-1 that affect Columbiformes are difficult to isolate in embryonated eggs but can replicate well in cell culture, viral isolation was attempted in both substrates for RT-qPCR positive samples (Capua and Alexander 2009). AOaV-1 can replicate in a variety of cell cultures of avian and non-avian origin, so African green monkey kidney cells clone E6 (VERO E6) were used in this work. Viral growth is normally accompanied by cytopathic effect. In AOaV-1 infection the cytopathic effect normally includes disruption of the cell monolayer and formation of syncytia (large multinucleate cells formed from the fusion of several cells).

Adherent VERO E6 cells were passed using the following protocol. After disposing the cell culture medium present in a T-25 flask, 2 ml of PBS was gently added to the cell monolayer to remove the remaining medium supplemented with foetal bovine serum. The PBS was then discarded, and the adherent cells were detached with trypsin at 37 °C for 10 minutes. The detached cells were resuspended in a small volume of cell culture medium, transferred to a conical tube, and centrifuged at 708 *x g* for 10 minutes. The supernatant was then discarded, and the cells were suspended in a minimal volume of growth medium. A new T-25 culture flask was prepared with 8 ml of cell culture medium with 10% of foetal bovine serum; ¼ of the pelleted cells were added to it and were incubated at 37 °C, 5% CO^2.

3.11. Rapid haemagglutination test

This method is based in the interaction between chicken red blood cells (RBCs) and HA activity and is performed with the allantoic fluid of the inoculated embryonated eggs. If the virus has replicated in the embryonated egg the allantoic fluid will contain virions with HA activity, when the allantoic fluid is mixed with RBCs the latter macroscopically aggregate.

In order to perform the rapid haemagglutination test, a drop of allantoic fluid and the same amount of 10% chicken RBC suspension were placed in a concave glass slide with Pasteur pipettes. The two fluids were mixed and observed for the presence of RBCs aggregates after 30-60 seconds at room temperature.

3.13. Visualisation of AOaV-1 with transmission electron microscopy

Brain tissue from 118-RIAS sample was submitted to transmission electron microscopy to observe if the virus was present in the brain of the bird.

The brain homogenate was first clarified by centrifugation at 1500 *x g*, 10 minutes. The supernatant was then ultracentrifuged at 30000 *x g*, for one hour at 5 ˚C (OptimaTM LE-80K, Beckman Coulter, U.S.A.). The supernatant was discarded, and the pellet was sent to the Institute of Molecular Medicine (Lisbon, Portugal) for microscopic analysis.

3.14. Phylogenetic analysis

In order to evaluate the identity of the nucleotide sequences obtained, BLAST (Basic local alignment search tool) search in the National Centre for Biotechnology Information database (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used, with an E-value cut-off of $<1x10^{-50}$, for nucleotide sequences. If a BLAST search was needed for other procedures, the same E-value cut-off was used.

The nucleotide sequences were aligned with the Muscle algorithm with the default parameters using MEGA X software (Kumar et al. 2018). The aligned sequences were manually corrected for optimal genetic similarity.

For phylogenetic inference of AOaV-1, the pilot complete fusion gene dataset for rapid genotype identification of isolates created by Dimitrov et al. (2019) was used along with eight isolates previously isolated in Portugal (Henriques et al. 2017) and two strains isolated from humans (Goebel et al. 2007; Kuiken et al. 2018). A sub-tree including the genotype of the isolates of this work and three isolates from Portugal was also built for phylogenetic inference within the genotype (Henriques et al. 2017). The accession number of the sequences in the pilot tree are listed in Appendix 4 whereas the accession numbers of the sequences in the sub-tree are listed in Appendix 5.

Maximum-likelihood methods were used to infer phylogenetic relationships and construct phylogenetic trees. Maximum Likelihood trees based on general time-reversible (GTR) model were constructed by using RaxML version 8.2.10 and raxmlGUI 2.0 with 1000 bootstrap replicates (Stamatakis 2014; Edler et al. 2020). Phylogenetic trees were visualized and visually arranged using Fig Tree v1.4.4.

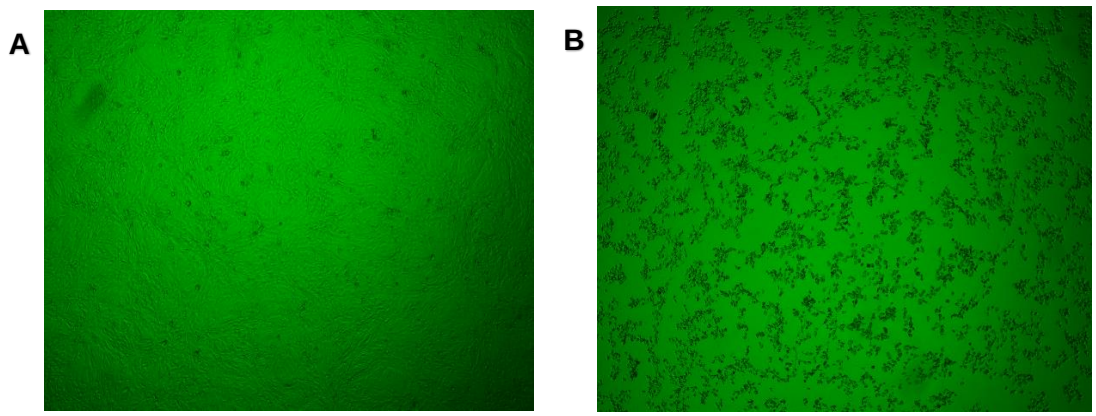
3.15. Seroneutralization test for WNV antibody detection and quantification

This method is based on the interaction of antibody-mediated virus neutralization and allows the titration of virus neutralizing antibodies, responsible for blocking the viral infection and replication in permissive animal cells.

The serum samples were inactivated at 56 °C for 30 min. Starting from a dilution titre of 1:2, serial 2-fold dilutions were made in microtiter plates, and 100 tissue culture infective doses (TCID) of Egypt 101 virus strain were added to each dilution. Thereafter, the mixtures were incubated at 37 °C for 1 h, and 10^5 Vero cells were added to each well. The plates were incubated at 37 °C 5% CO_2 from 3 to 5 days. Starting from the third day after incubation, the plates were checked for cytopathic effect, and the antibody titre was defined as the reciprocal of the highest dilution of the serum that showed 100% neutralization. A positive result is

represented by an intact monolayer, while a negative result is presented by a destroyed monolayer (figure 6). A titre equal or greater to 1/16 was considered specific for WNV.

Figure 6 - Results from seroneutralization test. An intact cell monolayer represents a positive result (A) while destroyed cell monolayer represent a negative result (B) (40x). Source: original.



3.16. Statistical analysis

A preliminary assessment of the database was performed with Microsoft Excel 2016® which allowed the detection of conflicting data, helped in the choice of the statistical tests to be used and in calculating the relative frequencies. Statistical analysis was conducted with The R Project for Statistical Computing, version 3.6.1, R packages 'exact2x2' version 1.6.5 and 'Rcmdr' version 2.7-1. Fisher's exact test was used to assess the association between order, species, migratory status, age, sex, region of the country and admission causes with the respective outcome. The test was evaluated with a statistical significance of 5% (α=0.05).

When pertinent, odds ratio (OR) was used to express the measure of association between each factor tested and the outcome with 95% confidence interval (95% CI). OR = 1, meaning that the studied factor does not affect the odds of having a positive test result. OR > 1, represents that the studied factor is associated with greater odds of having a positive test result. OR < 1, meaning that the studied factor is associated with lower odds of having a positive test result. OR values can range from 0 to infinity (Inf).

For data analysis simplicity, birds were considered adults when they had been born in or before the previous year and juveniles when the sample was taken in the same year that the bird had hatched. Whenever the bird's age was unknown the animal was excluded from the Fisher's exact test used to assess the association between age and respective outcome. When considered interesting and when bird age was recorded with the EURING nomenclature (EURING 2020) (Appendix 6), more distinction between bird age was made to better characterize the individual.

Region of the country was composed of three categories, North, Centre and South, based on the official Nomenclature of Territorial Units for Statistics (NUTS) II with some alterations to facilitate and confer more robustness to the statistical analysis (EC 2020). Lisbon Metropolitan area, Alentejo and Algarve that are separate NUTS II were merged as a single South region since they share similar ecological traits and landscapes. Moreover, when a bird's sex was unknown the animal was also excluded from the Fisher's exact test used to assess the association between sex and respective outcome. Migratory status was also altered, and individuals of species that can either migrate or not were all assigned as migratory.

Regarding the causes of admission, traumatic events such as caught in trap, shot, run over, collision, electrocution, predation by domestic animals, among others were all included in one single trauma category.

4. RESULTS

4.1. Molecular analysis

4.1.1. Characterization of the sampled population

Birds, belonging to 45 different species and 11 different orders (Table 1) were analysed for the presence of WNV, USUV, IAV and AOaV-1 by molecular methods. Within the analysed samples, the most prevalent order was Strigiformes with 26.37% (48/182) with the most prevalent species being tawny owl (*Strix aluco*) with 41.67% (20/48).

Most samples were collected from animals from the South, representing 75.27% (137/182) of the sample. The North region represented 17.03% (31/182) of the sample, the Centre represented 1.65% (3/182) and 6.04% samples were from unknown region (11/182).

Most animals were admitted to wildlife rehabilitation centres due to trauma, representing 47.80% (87/182) of the sample. The remaining causes for admission included, paretic syndrome [13.74% (25/182)]; nestlings [12.09% (22/182)]; weakness [6.59% (12/182)], suspicion of intoxication [5.49% (10/182)], illegal captivity [2.20% (4/182)], dystocia [0.55% (1/182)] and unknown causes [11.54% (21/182)].

Regarding the migratory status, 62.64% (114/182) individuals were resident (non-migrant) species, while 37.36% (68/182) were migrant species.

Regarding age, 67.03% (122/182) of individuals were adults, 31.32% (57/182) were juveniles and 1.65% (3/182) were from undefined age.

Regarding the sex, 41.21% (75/182) were from undefined sex, 31.87% (58/182) were males and 26.92% (49/182) were females.

Table 1 - Absolute frequencies (N) and relative frequencies (%) of orders and species tested for the presence of AOaV-1 with molecular tests. Species with an asterisk (*) are exotic species.

Order	Order frequence		Species	Species frequence within order	
	N	%		N	%
Accipitriformes	29	15.93	*Accipiter gentilis*	1	3.45
			Accipiter nisus	4	13.79
			Aegypius monachus	1	3.45
			Buteo buteo	15	51.72
			Circaetus gallicus	1	3.45
			Hieraaetus pennatus	4	13.79
			Milvus migrans	2	6.90
			Pernis apivorus	1	3.45
Anseriformes	7	3.85	*Alopochen aegyptiaca**	1	14.29
			Spatula clypeata	4	57.14
			Anas platyrynchos	1	14.29
			Mareca strepera	1	14.29
Apodiformes	6	3.30	*Apus apus*	2	33.33
			Apus pallidus	4	66.67
Charadriiformes	33	18.13	*Chroicocephalus ridibundus*	2	6.06
			Gallinago gallinago	1	3.03
			Larus audouinii	1	3.03
			Larus fuscus	14	42.42
			Larus michahellis	15	45.45
Ciconiiformes	7	3.85	*Ciconia ciconia*	7	100
Columbiformes	8	4.40	*Streptopelia decaocto*	8	100

Table 1 – Continuation.

Order	Order frequence		Species	Species frequence within order	
	N	%		N	%
Falconiformes			*Falco naumanni*	1	14.29
			Falco peregrinus	1	14.29
			Falco tinnunculus	5	71.43
Gruiformes	5	2.75	*Fulica atra*	4	80
			Gallinula chloropus	1	20
Passeriformes	17	9.34	*Acridotheres cristatellus**	1	5.88
			Corvus corax	1	5.88
			Corvus corone	3	17.65
			Erithacus rubecula	1	5.88
			Fringilla coelebs	1	5.88
			Garrulus glandarius	3	17.65
			Hirundo rustica	1	5.88
			Pica pica	1	5.88
			Sylvia atricapila	1	5.88
			Sturnus unicolor	1	5.88
			*Taeniopygia guttata**	1	5.88
			Turdus merula	2	11.76
Strigiformes	48	26.37	*Asio flammeus*	2	4.17
			Asio otus	1	2.08
			Athene noctua	17	35.42
			Bubo bubo	3	6.25
			Strix aluco	20	41.67
			Tyto alba	5	10.42
Suliformes	15	8.24	*Morus bassanus*	15	100

4.1.2. Analysis of the amplification products

A total of 60 samples were analysed for the presence of WNV by RT-qPCR and 122 by conventional RT-PCR, totalizing 182 samples analysed for WNV. All samples without amplification products or with amplification of unspecific products, will not be mentioned because they were not considered relevant for discussion.

A total of 88 samples were analysed for the presence of USUV by RT- and 94 by conventional RT-PCR, totalizing 182 samples analysed for USUV.

A total of 182 samples were analysed for the presence of IAV and AOaV-1 by RT-qPCR.

4.1.2.1. Amplification products of WNV, USUV, AOaV-1 and IAV by RT-qPCR

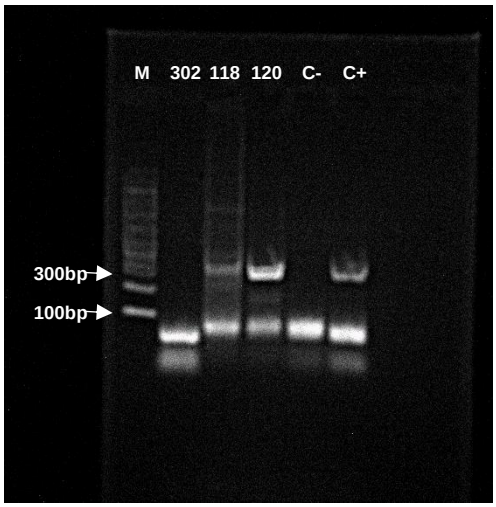
No amplification products were obtained for WNV, USUV and IAV.

The nucleic acid amplification of AOaV-1 by RT-qPCR resulted in one positive amplification product (Ct value =15.09) from a pool containing samples 118-RIAS, 119-RIAS and 120-RIAS, belonging to three *S. decaocto* specimens. The three samples were then subjected to RT-PCR individually.

4.1.2.2. Amplification products using specific primers for AOaV-1 and sequencing results

The pool containing samples 118-RIAS, 119-RIAS and 120-RIAS was subjected to RT-PCR targeting the F-gene. Samples 118-RIAS and 120-RIAS yield two amplicons (figure 8) with the expected size of 362 bp for AOaV-1 and sequencing confirmed its specificity. Sample 119-RIAS did not yield any amplicons with the expected size and was considered negative.

Figure 7 - Amplification products of three samples. Samples 118-RIAS and 120-RIAS have a positive result while sample 302-LxCRAS has a negative result. Source: original.



M: weight size marker; C-: negative control; C+: positive control.

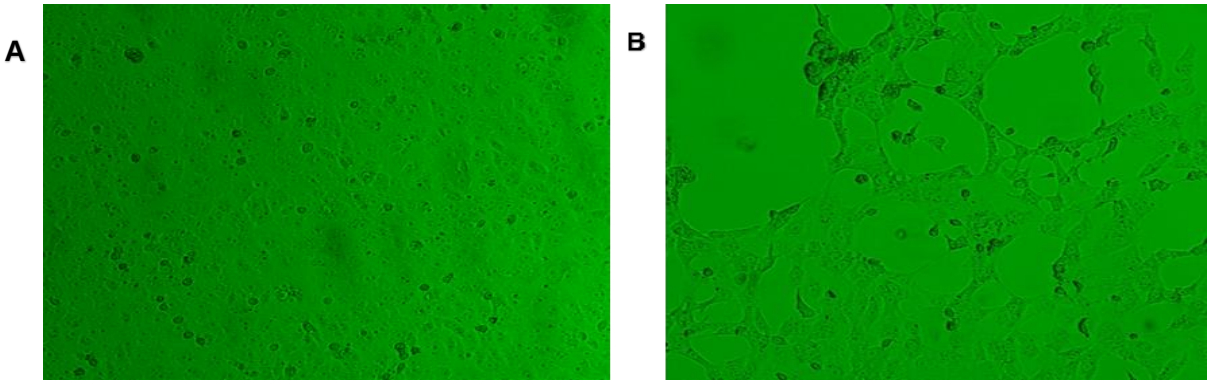
4.1.2.3. Viral isolation in chicken embryonated eggs and in cell culture

Viral isolation in chicken embryonated eggs of samples 118-RIAS and 120-RIAS was attempted. On the seventh day, allantoic fluid was collected from all the live embryos of each sample and tested by the rapid haemagglutination test. Sample 118-RIAS displayed haemagglutinating activity only in the first egg passage (one egg out of five), while sample 120-RIAS did not have haemagglutinating activity in neither of the three egg passages. Samples were collected and, since both samples were negative to the rapid haemagglutination test and some strains of AOaV-1 that affect Columbiformes are difficult to isolate in embryonated eggs, viral isolation in cell culture was attempted for both samples.

In viral isolation in cell culture cytopathic effect, such as disruption of the cell monolayer and formation of syncytia was observed in sample 120-RIAS, as seen in figure 7, which was AOaV-1 positive by RT-qPCR (Ct value = 20.40). In sample 118-RIAS cell culture occurred contamination, and new viral isolation from cell culture was not attempted. Instead, the allantoic fluid from sample 118-RIAS was subjected to RT-PCR analysis since some studies have reported that some strains of pigeon paramyxovirus type 1 (PPMV-1) may test negative by rapid haemagglutination test (King 1996). Sample 118-RIAS tested positive by RT-qPCR (Ct value = 4.56).

Samples from both sample 118-RIAS egg passage and sample 120-RIAS cell culture were collected and kept at -80˚C in the laboratory for future studies.

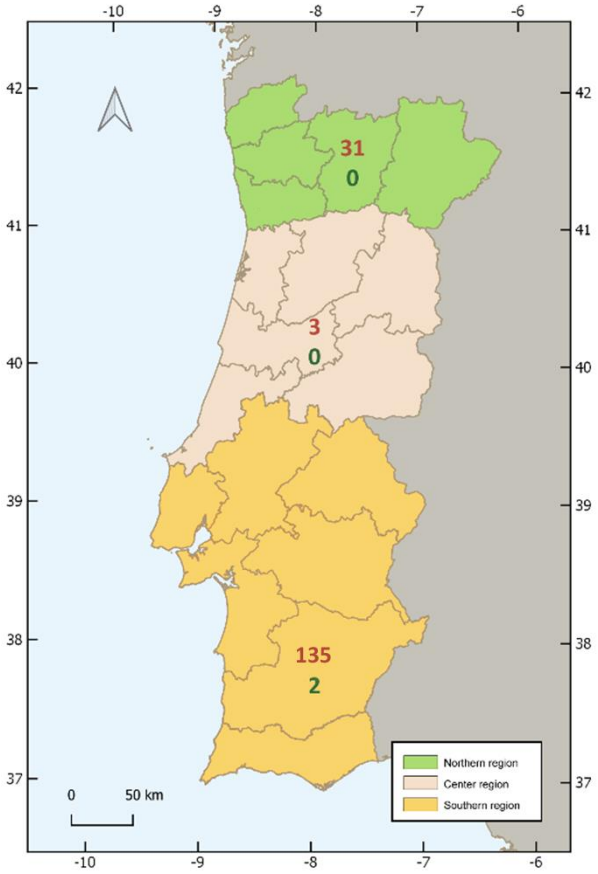
Figure 8 - Cell cultures. (A) intact monolayer of sample 120-RIAS at day 1 and (B) disrupted monolayer of sample 120-RIAS at day 5 (100x). Source: original



4.1.3. Analysis of the molecular results considering the studied population

In the studied population two samples were positive for AOaV-1 and none were positive for WNV, USUV and IAV. The number of positive and negative samples per region is represented in figure 9.

Figure 9 - Number of AOaV-1 positive and negative samples per region. Green numbers represent positive samples while red numbers represent negative samples. 11 negative samples were from unknown region and are not represented on the map. Source: original.



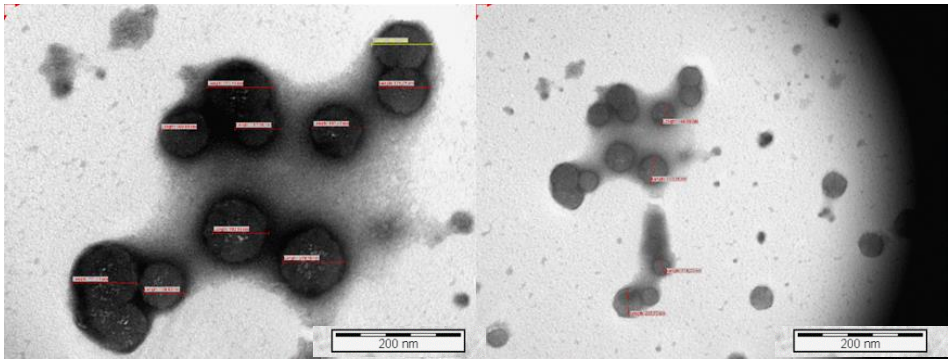
Two *S. decaocto* were positive for AOaV-1. The first one, sample 118-RIAS, was an adult male (with over one year of age) found dead in Olhão, which at necropsy was in the early stages of putrefaction and presented cachexia.

The second one, sample 120-RIAS, was also an adult male (over two years of age) found in Olhão, euthanised due to a left femoral, radial and ulnar fracture. At necropsy it presented cachexia and hepatomegaly.

4.1.4. Visualisation of AOaV-1 by transmission electron microscopy

Viral particles of pleomorphic or roughly spherical shape, measuring between approximately 116 to 223 nm of diameter were found in the brain tissue of sample 118-RIAS by transmission electron microscopy as seen in figure 10. These morphological characteristics are compatible with AOaV-1, since literature describes AOaV-1 particles as pleomorphic, roughly spherical, or filamentous shaped and measuring between 100 to 500 nm of diameter (Catroxo et al. 2012).

Figure 10 - Viral particles compatible with AOaV-1 view in electronic microscope. Source: iMM.



4.1.5. Phylogenetic analysis and nucleotide sequence analysis

The comparison between the nucleotide sequences from samples 118-RIAS and 120-RIAS did not identified point mutations between isolates, which suggests that the two viruses originated from a common ancestor.

The primers selected for AOaV-1 amplify a partial sequence of the F gene of 362 bp, translated into the aminoacidic residues 54 to 152 of the F protein. After translation of the partial coding sequence of the F protein, both strains contained the motif R-R-Q-K-R*F at the fusion protein cleavage site, between residues 113-117. This motif is associated with virulent viruses, due to the presence of three arginine (represented by R) between residues 113 and 116 and a phenylalanine (represented by F) at residue 117.

A search in the NCBI database was made to look for strains of AOaV-1 isolated from birds in Portugal, to compare the phylogenetic relationships of the strains characterized in our work and strains previously isolated in Portugal. Eight AOaV-1 strains previously characterized from Portugal, between 1998 and 2015, were found. A BLAST analysis was performed to compare these strains with the strains of this work and evaluate their correspondent E-value in order to investigate their inclusion in the phylogenetic analysis. All the sequences had an E-value $<1x10^{-50}$, and all were included in the phylogenetic analysis. The respective host and year of each strain can be consulted in Table 2.

Table 2 - Nucleotide sequences of portuguese AOaV-1 strains found in the NCBI database used in this work.

Acession number	Host	Year
KX831612	*Pigeon*	2015
KX831613	*Pigeon*	2015
KX831614	*S. turtur*	2015
AF503647	*Pigeon*	1998
AY471777	*Pigeon*	1999
AY4711775	*Pigeon*	1999
AY471776	*Pigeon*	1998
AY471774	*Pigeon*	2001

To infer the phylogenetic relationships of the strains, a Maximum Likelihood phylogenetic analysis was performed. Dimitrov et al. (2019), suggest that when genotyping a new isolate, a phylogenetic analysis with 125 pilot sequences should be made. Considering this, the sequences used included sequences from the pilot tree constructed by Dimitrov et al. (2019), the eight Portuguese isolates (Table 2), two sequences isolated from humans, with NCBI accession numbers EF555096 and KJ544861 (Goebel et al. 2007; Kuiken et al. 2018) and the two nucleotide sequences obtained in this work. Sequences retrieved from humans' isolates were used to infer the phylogenetic relationship between these sequences and the sequences characterized in this work. A total of 137 sequences were used, with accession numbers stated in Appendix 4. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. A bootstrap cut-off value of 70% was used. The retrieved phylogenetic tree can be seen in figure 11.

Tree analysis shows the class II 21 genotypes described by Dimitrov et al. (2019). Samples 118-RIAS and 120-RIAS clustered within genotype XXI, with a bootstrap value of 97%, together with three previous isolates from Portugal, namely KX831612, KX831613 and KX831614. The other five isolates from Portugal and both human isolates clustered within genotype VI, the closest phylogenetically genotype to the XXI genotype. Portuguese isolates (AF503647, AY471777, AY4711775, AY471776 and AY471774) and the human isolate EF555096 clustered within subgenotype VI.2.1.1.1. and human isolate KJ544861 clustered within subgenotype VI.2.1.1.2.2.

A sub-tree to infer phylogenetic relationships within the XXI genotype was also constructed (figure 12). The sequences set included 51 XXI genotype sequences, the two rooting sequences (accession numbers JX915243 and Z12111) used by Dimitrov et al. (2019), the three Portuguese nucleotide sequences that also clustered within genotype XXI (KX831612, KX831613 and KX831614) and the two sequences newly characterized in this work (the 58 sequences are specified in Appendix 5).

Figure 11 – Class II phylogenetic tree based on F gene. Inferred using RaxML version 8.2.10 and the Maximum Likelihood method based on the General Time Reversible model with 1000 bootstrap replicates. The tree was edited using FigTree V1.4.4. The tree with the highest log likelihood (-29200.239183) is shown. The analysis involved 137 nucleotide sequences. The respective genotype of each strain is represented in Roman numerals. The taxa names includes the genotype, the GenBank accession number, host name, country and year of isolation. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values ≥75 are indicated next to the branches.

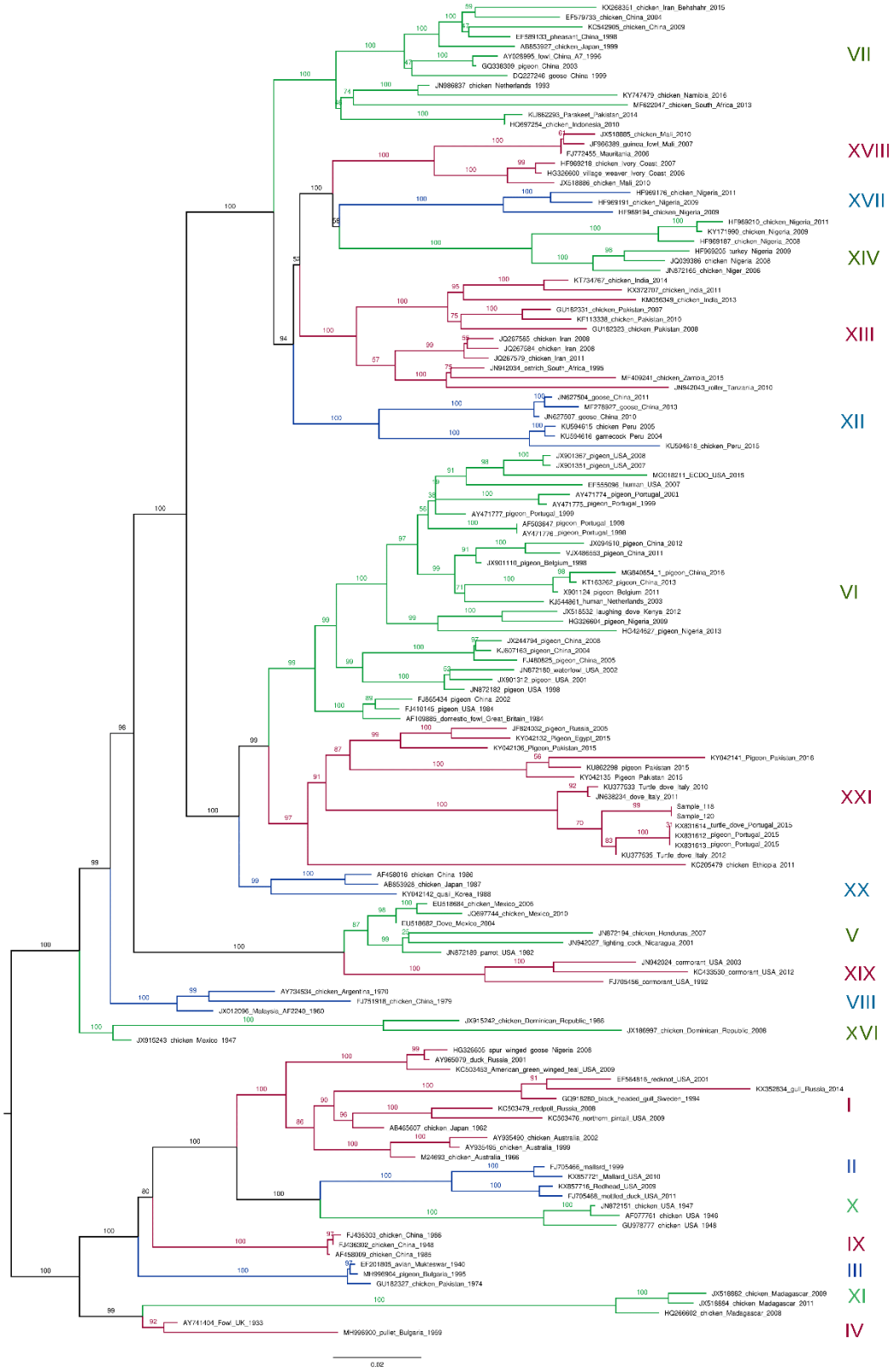
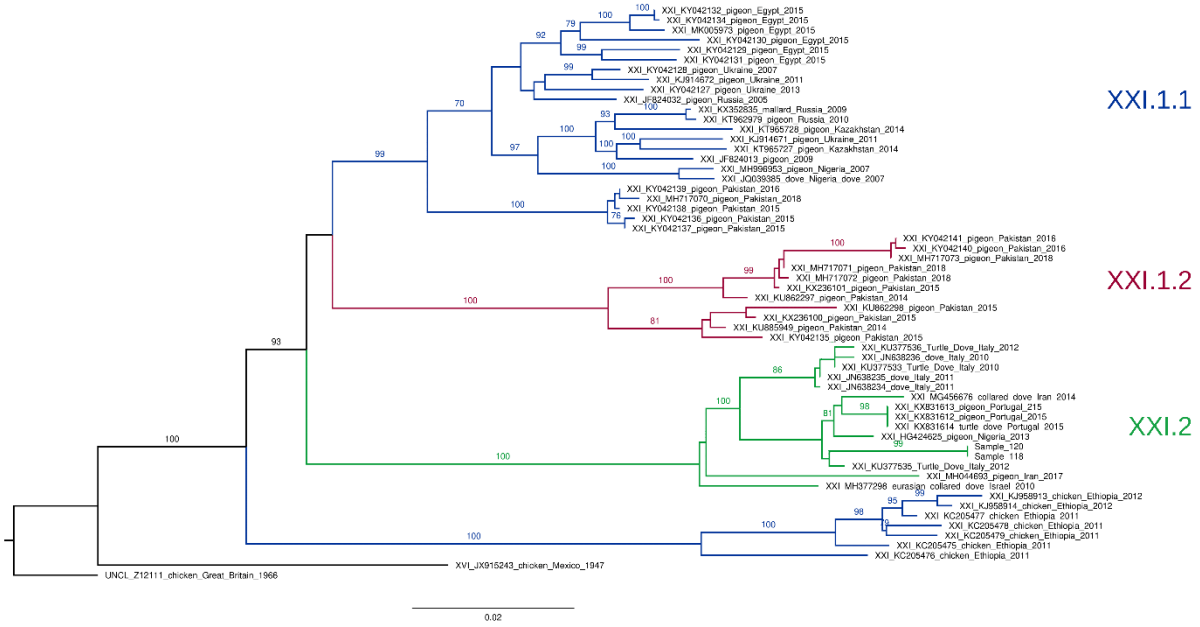


Figure 12 – Genotype XXI phylogenetic tree based on F gene. Inferred using RaxML version 8.2.10 and using the Maximum Likelihood method based on the General Time Reversible model with 1000 bootstrap replicates. The tree was edited using FigTree V1.4.4. The tree with the highest log likelihood (-8716.958219) is shown. The analysis involved 58 nucleotide sequences. Strains JX915243 and Z12111 were included as an outer group for rooting purposes. The respective genotype of each strain is represented in Roman numerals. The taxa names includes the genotype, the accession number, host name, country of isolation and year of isolation. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values ≥75 are indicated next to the branches.



Genotype XXI includes viruses isolated from chickens and Columbiformes in different Asian, European, and African countries between 2005 and 2016 that were previously assigned as genotype VI viruses (Dimitrov et al. 2019). Three subgenotypes exist within genotype XXI, namely genotypes XXI.2, XXI.1.1, and XXI.1.2 corresponding to former subgenotypes VIi, VIg, and Vim (Dimitrov et al. 2019). Almost all strains comprised in this genotype were obtained from Columbiformes, such as pigeons, *S. turtur*, *S. decaocto* and undefined dove species. Genotype XXI also comprises one strain from a mallard (*Anas platyrhynchos*) obtained in Russia in 2009 and some strains isolated from chickens in Ethiopia between 2011 and 2012 that previously formed a different subgenotype, VIl, but due to insufficient number of epidemiologically independent isolates were assigned to the lower order, namely XXI. All these strains contain a motif at the fusion protein cleavage site associated with virulent viruses, being either R-R-K-K-R*F or R-R-Q-K-R*F, posing a threat to poultry species (Aldous and Alexander 2001).

Samples 118-RIAS and 120-RIAS clustered within subgenotype XXI.2, with 13 other strains, namely JN638234, JN638235, JN638236, KU377533, KU377536, MG456676, HG424625, KX831612, KX831613, KX831614, KU377535 and MH377298. These strains were isolated from a pigeon from Nigeria, and *S. turtur* and *S. decaocto* from Italy, Nigeria, Portugal, Iran and Israel. Within this subgenotype, two groups with a strong bootstrap support

can be observed. JN638234, JN638235, JN638236, KU377533 and KU377536 Italian strains isolated from undefined dove species and *S. turtur* from 2010 to 2012 grouped together with a bootstrap value of 86%. MG456676, HG424625, KX831612, KX831613 and KX831614 Nigeria, Iran and Portugal isolates from pigeons and a *S. decaocto* 2013 to 2015 also grouped together with a bootstrap value of 81%.

4.2. Serological analysis

4.2.1. Characterization of the sampled population

73 animals were analysed for the presence of WNV antibodies by seroneutralization test, belonging to nine different orders and 23 different species (Table 3).

Within the analysed samples, the most prevalent order was Strigiformes with 34.25% (25/73), the most prevalent species being the *S. aluco* with 64% (16/25).

Most samples were collected from animals from the North of Portugal, representing 45.21% (33/73) of the sample. The South region represented 39.73% (29/73) of the samples, the Centre represented 9.59% (7/73) and 5.48% samples where from an unknown region (4/73).

The most prevalent cause for admission was trauma (including shot, caught in trap, caught in fishing hook, run over, collision, electrocution, predation by domestic animals, among others less represented traumatic causes) representing 45.21% (33/73) of the sample. The remaining causes included nestlings [21.92% (16/73)]; paretic syndrome [17.81% (13/73)]; illegal captivity [2.74% (2/73)]; suspicion of intoxication [2.74% (2/73)]; weakness [1.37% (1/73)]; dystocia [1.37% (1/73)] and unknown causes [6.85% (5/73)].

Regarding their migratory status, 68.49% (50/73) of individuals were resident (non-migrant) species, while 31.51% (23/73) were migrant species.

Regarding age 72.60% (53/73) of individuals were adults and 27.40% (20/73) were juveniles.

Regarding the sex, 78.08% (57/73) were of undefined sex, 13.70% (10/73) were females and 8.22% (6/73) were males.

Table 3 - Absolute frequencies (N) and relative frequencies (%) order and species tested for the presence of WNV with serological tests.

Order	Order frequency		Species	Species frequency within order	
	N	%		N	%
Accipitriformes	21	28.77	*Accipiter gentilis*	3	14.29
			Aegypius monachus	1	4.76
			Accipiter nisus	3	14.29
			Buteo buteo	8	38.10
			Circaetus gallicus	1	4.76
			Hieraaetus pennatus	2	9.52
			Milvus migrans	1	4.76
			Milvus milvus	2	9.52
Anseriformes	1	1.37	*Alopochen aegyptiaca*	1	100
Charadriiformes	16	21.92	*Larus fuscus*	11	68.75
			Larus michahellis	5	31.25
Ciconiiformes	4	5.48	*Ciconia ciconia*	4	100
Columbiformes	1	1.37	*Streptopelia decaocto*	1	100
Falconiformes	1	1.37	*Falco peregrinus*	1	100
Passeriformes	3	4.11	*Corvus corone*	1	33.33
			Garrulus glandarius	1	33.33
			Pica pica	1	33.33
Pelecaniformes	1	1.37	*Ardea cinerea*	1	100
Strigiformes	25	34.25	*Asio flammeus*	1	4
			Athene noctua	4	16
			Bubo bubo	1	4
			Strix aluco	16	64
			Tyto alba	3	12

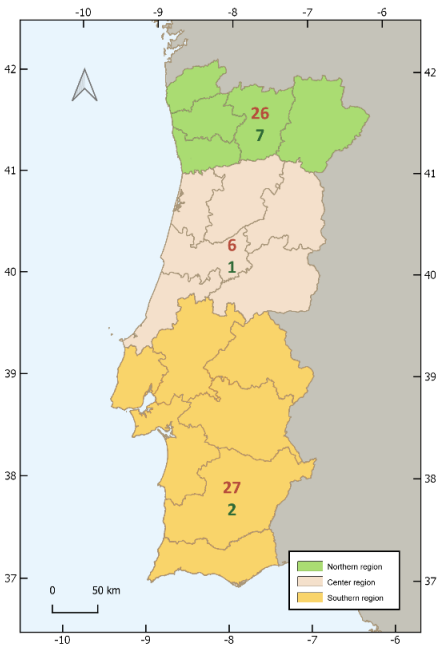
4.2.2. Serological analysis results

From the 73 tested samples, ten were positive for the presence of neutralizing antibodies against WNV (13,7% positivity) (Table 4) with antibody titers ranged from 1:16 to 1:64. Of these positive samples, seven were from the Northern region, one from the Center and two from the Southern region as seen in figure 13.

Table 4 - Number of WNV positive samples through serological test by species.

Order	Family	Species	VNT WNV positive				
			N	Titres			Wildlife rehabilitation centre
				1:16	1:32	1:64	
Accipitriformes	Accipitridae	Cinereous vulture (*Aegypius monachus*)	1	1			CRAS-HVUTAD
		Eurasian sparrowhawk (*Accipiter nisus*)	1		1		CRAS-HVUTAD
		Common buzzard (*Buteo buteo*)	4	3	1		3 from CRAS-HVUTAD 1 from LxCRAS
		Short-toed snake eagle (Circaetus gallicus)	1	1			CRAS-HVUTAD
		Booted eagle (*Hieraaetus pennatus*)	2			2	CRAS-HVUTAD
Ciconiiformes	Ciconiidae	White stork (*Ciconia ciconia*)	1	1			LxCRAS
		Total	10	6	2	2	

Figure 13 - Number of WNV serological positive and negative samples per region. Green numbers represent positive samples while red numbers represent negative samples. Four negative samples were from unknown region and are not represented on the map. Source: original.



4.2.3. Analysis of the serological results considering the studied population

Eight of the ten individuals with positive samples were from CRAS-HVUTAD while the other two were from LxCRAS.

Sample 209-CRAS-HVUTAD belonged to a short-toed snake eagle (*C. gallicus*) from undefined sex with age 4 (born in 2017 or earlier) that was found in Vila Nova de Gaia, Portugal due to trauma. There were no ongoing clinical signs compatible with WNV infection or WNV infection sequelae.

Sample 210-CRAS-HVUTAD belonged to a male Eurasian sparrowhawk (*A. nisus*) with age 4 (born in 2017 or earlier) found in Boticas, Vila Real, Portugal due to trauma. There were no ongoing clinical signs compatible with WNV infection or sequelae.

Sample 213-CRAS-HVUTAD belonged to male common buzzard (*B. buteo*) with age 5 (born in 2018) found in Peso da Régua, Vila Real, Portugal due to gunshot, presenting multiple fractures and neurological signs such as head tilt and anisocoria. Although the bird presented with neurological signs compatible with WNV infection, it was likely that these neurological signs were due to the gunshot, since the lead bullet had lodged in the occipital region.

Sample 214-CRAS-HVUTAD belonged to a female *B. buteo* with age 5 (born in 2018) found in Vila Pouca de Aguiar, Vila Real, Portugal due to multiple trauma. There were no ongoing clinical signs compatible with WNV infection or sequelae.

Sample 232-CRAS-HVUTAD belonged to a male *B. buteo* with age 4 (born in 2017 or earlier) found in Venda Nova de Montalegre, Vila Real, Portugal. The cause of admission was unknown, but the animal presented with Horner's syndrome, airsacculitis, increased lung radiopacity, and haematomas in the left ear and oral cavity. The bird presented neurological signs compatible with WNV infection, but respiratory signs are not commonly reported in WNV. Clinical signs were more compatible with the occurrence of a traumatic event.

Sample 237-CRAS-HVUTAD belonged to a cinereous vulture (*A. monachus*) of undefined sex with age 5 (born in 2018) found in Castelo Branco, Portugal due to trauma. There were no ongoing clinical signs compatible with WNV infection or sequelae.

Sample 243-CRAS-HVUTAD belonged to a male booted eagle (*H. pennatus*) with age 6 (born in 2016 or earlier) found in Montalegre, Vila Real, Portugal due to trauma. There were no ongoing clinical signs compatible with WNV infection or sequelae.

Sample 244-CRAS-HVUTAD belonged to a *H. pennatus* of undefined sex with age 6 (born in 2016 or earlier) found in Vila Nova de Gaia, Porto, Portugal due to trauma. There were no ongoing clinical signs compatible with WNV infection or sequelae.

Sample 327-LxCRAS belonged to a white stork (*C. Ciconia*) of unknown sex with age 4 (born in 2018 or earlier) found in Santarém, Portugal with a suspected intoxication. There were no ongoing clinical signs compatible with WNV infection or sequelae.

Sample 328-LxCRAS belonged to a *B. buteo* of undefined sex with age 6 (born in 2016 or earlier) found in Sintra, Lisbon, Portugal due to gunshot. There were no ongoing clinical signs compatible with WNV infection or sequelae.

4.3. Statistical analysis

4.3.1. Statistical analysis regarding AOaV-1 infection

The association between bird order and outcome in the AOaV-1 molecular test was statistically significant (p<0.01). Animal orders were tested separately to assess whether each order was significantly associated with each outcome. Belonging to the order Columbiformes was significantly associated with having a more frequent “positive AOaV-1 result in molecular test” [OR Inf, CI 95%: 6.79-Inf; (p<0.01)]. All other relations between each order and the outcome were not statistically significant (Appendix 7).

Animal species were also tested separately to assess whether each species was significantly associated with the outcome. Belonging to the species *S. decaocto* was significantly associated with having a more frequent “positive AOaV-1 result in molecular test” [OR Inf, CI 95%: 6.79-Inf; (p<0.01)]. The order Columbiformes was only composed by *S. decaocto* specimens, which explains why the results from the Fisher’s exact test and OR from the association between Columbiformes and molecular test outcome and from the association between *S. decaocto* and molecular test outcome the same. All other relations between each species and the outcome were not statistically significant (Appendix 8).

The respective associations between region of the country where the animal was found, age, sex, cause of admission or migratory status, and outcome in AOaV-1 molecular test were not statistically significant (Table 5).

Table 5 - Fischer's exact test results for the selected biological factors and outcome in AOaV-1 molecular test.

Factor	Fisher’s exact test
Bird order	P<0.01
Region of the country where the animal was found	p=1
Age	OR = Inf, CI 95%: 0.13-Inf; (p=1)
Sex	OR = Inf, CI 95%: 0.24-Inf; (p=0.50)
Cause of admission	p=0.52
Migratory status	OR = Inf, CI 95%: 0.17-Inf; (p=0.53)

4.3.1. Statistical analysis regarding WNV infection

The association between bird order and outcome in WNV serological test was statistically significant (p<0.01). Animal orders were tested separately to assess whether each order was significantly associated with each outcome. Belonging to the order Accipitriformes was significantly associated with having a more frequent positive AOaV-1 result in molecular test [OR = 35.85, CI 95%: 4.76-840.82; (p<0.001)] while belonging to the order Strigiformes was significantly associated with having a more frequent negative AOaV-1 result in molecular test [OR = 0, CI 95%: 0-0.76; (p<0.05)]. All other relations between each order and the outcome were not statistically significant (Appendix 9).

The association between bird species and outcome in WNV serological test was statistically significant (p<0.01). Animal species were also tested separately to assess whether each species was significantly associated with the outcome. Belonging to the species *B. buteo* was significantly associated with having a more frequent "positive AOaV-1 result in molecular test" [OR = 9.31, CI 95%: 1.80-49.78; (p<0.05)]. Belonging to the species *H. pennatus* was also significantly associated with having a more frequent "positive AOaV-1 result in molecular test" [OR = Inf, CI 95%: 1.92-Inf; (p<0.05)]. All other relations between each species and the outcome were not statistically significant (Appendix 10).

The association between sex and positive outcome in the WNV serological test was statistically significant [OR = 14, CI 95%: 1.14-480.95; (p<0.05)].

The respective associations between age, region of the country where the animal was found, cause of admission and migratory status with a positive outcome in WNV serological test were not statistically significant (Table 6).

Table 6 - Fischer's exact test results for the between selected biological factors and outcome in WNV serological test.

Factor	Fisher's exact test
Bird order	p<0.01
Region of the country where animal was found	p=0.23
Age	OR = 0, CI 95%: 0-1.09; (p = 0.05)
Sex	OR = 14, CI 95%: 1.14-480.95; (p<0.05)
Cause of admission	p=0.08
Migratory status	OR = 0.65, CI 95%: 0.14-2.73; (p=0.72)

5. Discussion

AOaV-1 is considered one of the most problematic infectious diseases in poultry and has been extensively studied in these species. However, little is known about the epidemiology of AOaV-1 in wild birds, the true impact that this disease can have in wild species' populations and as a spill over to domestic populations (Alexander 2007). In the Iberian Peninsula, very few prevalence studies of AOaV-1 infection in wild birds have been reported and, to the authors' knowledge, there are no reported prevalence studies in the wild bird populations of Portugal. In Spain, Napp et al. (2017) reported a prevalence of 50% (74/148) AOaV-1 RNA positive wild birds. The detection was based almost exclusively on passive surveillance in birds that died in mortality events, which could have led to biased results. Species that are more susceptible to AOaV-1 infection and present with more severe clinical signs probably are more prone to die and, thus, AOaV-1 prevalence in these studies can be higher than expected. It is also interesting to note that of the 79 mortality events which occurred during the study's period, 35 were attributed to AOaV-1 and, of these, 33 were associated with *S. decaocto* populations. Another study, by Maldonado et al. (1994), found a 1.4% prevalence of (5/361) AOaV-1 neutralizing antibodies in wild birds, with the five individuals belonging to the Anseriformes order (*A. platyrynchos, M. strepera* and *Netta ruffina*). The study sampled 317 aquatic species, and 44 non-aquatic species, with 3 samples belonging to wood pigeon (*Columba palumbus*) (Columbiformes).

In our study, AOaV-1 infection was detected in two *S. decaocto* from Olhão, Faro (sample 118-RIAS and sample 120-RIAS). The overall AOaV-1 prevalence was 1.10% (2/182) and the prevalence within the species was 25% (2/8). Although the overall prevalence was similar to that of the Maldonado et al. (1994) study, the birds' order where AOaV-1 was detected were not the same. Maldonado et al. (1994) described AOaV-1 infection in five Anseriformes while in our study we only found AOaV-1 infection in Columbiformes. In our study, only 60 aquatic species were sampled, and, of these, only 7 were Anseriformes, compared to 317 aquatic species sampled by Maldonado et al. (1994) so it is possible that the discrepancy between the number of aquatic birds sampled contributed to the lack of more positive samples in the present study. Our study's prevalence is very different from that of the study of Napp et al. (2017), but is important to note, as said earlier, that Napp et al. (2017) collected samples through passive surveillance mainly associated to mortality events, which can possibly lead to a biased result. In their study, 50.7% of samples were collected from *S. decaocto*, and 33 of the 35 mortality events were associated with *S. decaocto* populations. It appears that there is an overrepresentation of *S. decaocto* specimens compared to other specimens and that the prevalence amongst *S. decaocto* specimens is considerably higher compared to other species, so it is very unlikely that these results represent the true prevalence

found across all bird species in the wild. This hypothesis is supported by our study, since the only Columbiform species we sampled was *S. decaocto* and belonging to the Columbiform order was associated with having a positive AOaV-1 result in the molecular test [OR Inf, CI 95%: 6.79-Inf; ($p<0.01$)]. It is possible that the association between Columbiform order and having a positive test would be less significant if samples from other species would have been collected, so assumptions made based on this result should be made with caution. However, the association between *S. decaocto* and having a positive test is clearer, with two of an universe of 182 birds being positive. This is supported by various studies, with the scientific community concisely detecting AOaV-1 in wild Columbiformes, mainly from *S. decaocto* and *S. turtur*, supporting the idea that an endemic panzootic exists in domestic Columbiformes which occasionally spills over to wild Columbiformes (Alexander 2011; Napp et al. 2017). Moreover, being an S. decaocto appears to be one of the main factors for having a positive AOaV-1 test result, since other biological factors, such as age, sex, cause of admission or migratory status did not have a statistically significant association.

S. decaocto is considered one of the biggest colonizer species of the vertebrate world. Before the 20th century the species could be found in Southern Asia, its original geographical range, but it had also spread to China and the Middle East. However, in the 20th century, it spread very rapidly towards the west, in the direction of Europe. Between 1930 and 1970 the species colonised an estimated 2.5 million km^2 in north-western Europe (Reino et al. 1998). In Portugal, the first known record dates to 1974 in Porto and since then its spread to the whole country, being mainly present in coastal areas (Santos Júnior 1979; Reino et al. 1998). Almost all European countries have been colonised by *S. decaocto* (Birdlife 2020). The study by Napp et al. (2017) found a correlation between the distribution of *S. decaocto* reported in Catalonia and the location of the mortality episodes caused by AOaV-1 detected in Catalonia between 2010 and 2016. With the results found in our study and other concisely reported detections of AOaV-1 infections in *S. decaocto*, one may infer if it is possible that an association between the distribution of *S. decaocto* or other Columbiformes and the mortality episodes caused by AOaV-1 exists. More studies regarding this subject and the true role of *S. decaocto* and other Columbiformes distribution should be made to access if the expansion of the species to new areas and their population increase could result in an increase in AOaV-1 outbreaks in domestic and wild birds.

Even if the role of *S. decaocto* in AOaV-1 epidemiology has yet to be explained, it is a fact that the detection of AOaV-1 in *S. decaocto* poses a risk not only to this species, but also to other wild species and poultry species. For example, *S. turtur* is an endangered columbiform closely related to *S. decaocto*, in which we have witnessed a decrease in number of breeding individuals of approximately 80% in Portugal, in the last years (Dias 2016). Scientific studies

have been suggesting that this decline is mainly due to habitat loss/modification, hunting, poaching and climate change. Reported cases of AOaV-1 infection in this species are documented; it is possible that the virus may also be an unnoticed threat to both species populations in Portugal. (Terregino et al. 2003; Bonfante et al. 2012).

The two AOaV-1 sequences found in our study were identical regarding the 362 bp nucleotide sequence. It is possible, that the birds were infected with the same strain, since they were caught in the same region. However, this would only be confirmed by whole genome sequencing. Both strains contained a motif at the fusion protein cleavage site associated with virulent viruses (R-R-Q-K-R*F). In individual 118-RIAS viral particles compatible with AOaV-1 were observed by transmission electron microscopy in the brain sample (Catroxo et al. 2012), pointing to a possible viral presence in the brain, which could have induced pathological changes in the CNS and possibly death. Controlled studies would be needed to prove that strains that circulate in Portugal could induce severe AOaV-1 infection that would lead to the death of wild birds and, specifically, wild doves species.

The phylogenetic analysis of the partial F gene coding nucleotide sequence from samples 118-RIAS and 120-RIAS showed they clustered within genotype XXI (figure 11 and 12), with another 54 sequences. Most of the strains comprised in this genotype were virulent strains obtained from Columbiformes between 2005 and 2018. The strains found in Porto Santo (KX831612, KX831613, KX831614) also grouped into genotype XXI (Henriques et al., 2017). Furthermore, it clustered within subgenotype XXI.2. with 13 other strains, namely JN638234, JN638235, JN638236, KU377533, KU377536, MG456676, HG424625, KX831612, KX831613, KX831614, KU377535 and MH377298. These strains were isolated in a pigeon from Nigeria, a *S. turtur* and *S. decaocto* from Italy, Nigeria, Portugal, Iran and Israel. Considering this, it is likely that both dove species and pigeons contribute to the maintenance of AOaV-1 and, to some extent, its' dispersal. Moreover, and considering the countries from whence these strains were isolated, it is likely that in the case of these strains, including those found by our study, *S. turtur* is the most likely to disperse the virus through long distances, since pigeons and *S. decaocto* are non-migrant species.

The known breeding distribution of *S. turtur* ranges from Europe (except for Iceland and some areas of northern Europe) to North Africa and central Asia. *S. turtur* has four subspecies, so it is important to note that their distribution areas are different across these countries and normally do not mix in breeding grounds. However, all four subspecies winter and mix in the same wintering grounds, in or near the Sahel region of Africa (figure 14). So, it is likely that some individuals from Portugal, Italy, Iran and Israel winter together in the Sahel region of Africa, that also incorporates Nigeria, and contaminate each other, passing through AOaV-1

strains between themselves. This allows for the dispersal of the virus not only between populations from *S. turtur* of different countries, but also to other birds, such as other Columbiformes, when they turn back to their breeding grounds and interact with local bird populations in the Sahel region (Fisher et al. 2018). For example, Snoeck et al. (2013), has characterized eight strains of AOaV-1 in eight pigeons sold at live-bird markets in Nigeria, access number HG424625, which clustered with the Italian strains, Porto Santo strains and our strain. Interestingly, Snoeck et al. (2013) also sampled 169 individuals from nine wild dove species from Nigeria that were negative for AOaV-1, suggesting that it may not yet be enzootic in wild birds of Nigeria, supporting the idea that *S. turtur* may have an important role in the dispersion of AOaV-1 in these countries. However, one cannot exclude the role of *S. decaocto*, pigeons and even other species in these long-distance disseminations through dispersion movements. Even so, if pigeons and *S. decaocto* do not have a role in the long-distance dissemination, they probably have a role in the maintenance of AOaV-1 in the breeding grounds of *S. turtur*. More studies should be made to understand these transmission dynamics and the roles that these species have in the epidemiology of AOaV-1 in these regions.

Figure 14 - World distribution of *Streptopelia turtur*. Yellow represents breeding grounds and blue represents wintering grounds. Source: Birdlife International 2020.

Moreover, AOaV-1 does not only affect wild animals. The presence of virulent strains of AOaV-1 in wild birds may also pose a threat to humans and domestic animals. Although most studies refer to AOaV-1 as causing only mild conjunctivitis, two worrying reports from 2002 and 2007 pose the question if there is greater danger associated with the infection than previously thought. Two immunocompromised individuals with AOaV-1 infection died, one in Belgium in 2002 and another in New York in 2007. In both cases there was evidence of extra-respiratory spread of AOaV-1 (Goebel et al. 2007; Kuiken et al. 2018). Moreover, Kuiken et al. (2017) also reported AOaV-1 causing respiratory disease in a non-human primate model,

suggesting that both human cases were, indeed, primary respiratory disease due to AOaV-1 infection. It is important to note that in the case of Belgium, although the patient died in 2002, it was only reported in 2018 due to a metagenomic analysis conducted in archived cell culture samples with an unexplained cytopathic effect (Kuiken et al. 2018). Considering this, it is not clear if AOaV-1 infection in humans is, indeed, very rare or if more cases of mortality or morbidity are not reported because suspicion of AOaV-1 infection is not placed due to its rarity. It is also possible that information regarding respiratory disease in humans is not yet widespread (Kuiken et al. 2017). Both strains found in humans were grouped into genotype VI, (figure 11) and both had a motif at the fusion protein cleavage site associated with virulent viruses, being either R-R-K-K-R*F in the strain characterized by Goebel et al. (2007) or R-R-Q-K-R*F in the strain characterized by Kuiken et al. (2018). Five Portuguese strains from pigeons (AF503647, AY471777, AY4711775, AY471776 and AY471774) also clustered within genotype V, which is not strange since this genotype is considered the most diverse among all AOaV-1 genotypes and has been isolated in all continents, except Antarctica (Dimitrov et al. 2016). Moreover, most of the strains that belong to this genotype have been isolated from Columbiformes thus suggesting that patients got infected by a Columbiform (Aldous et al. 2004; Dimitrov et al. 2019). Although these strains do not belong to the same genotype as the strains from our work, it is possible that genotype XXI strains also have the potential to infect and cause respiratory disease in humans. Human infections can occur due to contact with wild Columbiformes, such as *S. decaocto* or *S. turtur* or their faeces, due to contact with synanthropic species, such as domestic pigeons or their faeces, due to contact with infected cadavers or by wind-borne dust, when a highly virulent virus is present in the columbiform populations (Alexander 1988). Veterinarians, nurses and technicians who treat synanthropic species or domestic pigeons, hunters and immunocompromised individuals must be cautious when contacting with these animals.

No outbreaks were reported in the poultry farms of Algarve in the last few years. This could be due to the fact that there are few poultry farms in the area or because the biosecurity measures taken by poultry farms are very efficient. Moreover, some authors have suggested that PPMV-1 infections can go undetected in chickens due to the low pathogenicity of these strains in chickens despite their fusion protein multi-basic cleavage site. (Dortmans et al. 2009; Zhan et al. 2020). We did not perform monoclonal antibodies testing specific for PPMV-1 so it was not possible to conclude if the strains characterized in our study were PPMV-1 or not. Even if not classified as PPMV-1, it is still possible that other AOaV-1 strains capable of infecting pigeons and other Columbiformes also go undetected in chickens. In fact, some studies have even reported strains found in *S. decaocto*, associated with mortality events in this species in Spain and Italy, that did not react with pigeon-specific mAb 161/617 and thus

were not considered a PPMV-1 (Bonfante et al. 2012; Napp et al. 2017). Furthermore, Henriques et al. (2017) also reported that during the outbreak of AOaV-1 in Porto Santo, AOaV-1 antibodies were detected by HI in 30.4% (14/42) of asymptomatic poultry. Considering this, it is possible that some strains of AOaV-1, such as the ones antigenically classified as PPMV-1, can go undetected due to the low virulence and absence of clinical signs or mortality in poultry (Dortmans et al. 2009; Zhan et al. 2020). More studies in poultry species within the areas where these strains were detected would be needed to access this hypothesis.

IAV is another important disease with zoonotic potential which occurs among wild birds worldwide. Waterfowl, in particular dabbling ducks, represent the main reservoir for IAV. Portugal is a high-risk area for the transmission of IAV between birds, since millions of birds migrate each winter to the country or use the East Atlantic flyway that passes through it, facilitating transmission between different avian species from different origins (Munster et al. 2007; Wallensten et al. 2007). To the authors' knowledge, two studies of IAV prevalence in wild birds recurring to molecular testing were made. The first one included 5691 samples collected between 2005 and 2009 and found a prevalence of 1.63% IAV positive samples in wild birds, with Anseriformes representing 62.57% of the total population and mallard (*A. platyrynchos*) representing 26.27% of the total population (Henriques et al. 2011). Positive samples were found in *A. platyrynchos* [5.5% (82/1495)], *Anas crecca* [1.5% (3/204)], *Aythia fuligula* [4.2% (1/24)], *Larus sp.* [0.6% (3/522)], *Phoenicopterus sp*. [0.9% (1/194)], *Perdix perdix* [0.9% (2/227)], *C. ciconia* [0.6% (1/178)]. Another study conducted in western Portugal between 2008 and 2009 analysed 1653 samples from six different species of waterfowl, of which *A. platyrynchos* represented 93.28%. This study had 4.4% overall positive cases, and identified eight different IAV subtypes (Tolf et al. 2012).

In Spain, a study has reported an IAV prevalence of 0.60% (7/1186) in samples collected between 2014 and 2015 in south-central Spain (Bárbara et al. 2017). Positive samples were from C. *ciconia* (5/689), *Larus sp.* (1/361) and *Bubulcus ibis* (1/116). Another study conducted in a northern Spain wetland compared two sampling periods: the first one, between 2007 and 2009, recorded a prevalence of 6.0% (44/667), and the second period, between 2012 and 2014, recorded a prevalence of 0.3% (8/2725) (Torrontegi et al. 2019). Host identification was possible in 48% (25/52) of the samples and *A. platyrynchos* represented 44% (23/52) of positive samples (Torrontegi et al. 2019).

In our study, all tested samples were negative for the presence of IAV RNA. The discrepancy between the present work IAV prevalence and the studies mentioned before seeming to relate to the sample size and species sampled. Our study only sampled 182 birds, while the studies cited above sampled between 1181 and 5691 birds, and thus, it was less

likely for our study to find positive samples. Moreover, in our study Anseriformes only represented 3.85% (7/182) and *A. platyrynchos* 0.55% (1/182) of samples while in two previous studies made in Portugal cited before, Anseriformes represented 62.57% and 100% of samples and *A. platyrynchos* represented 26.27% and 93.28% of total samples, with an overall prevalence IAV infection of 1.6% in the first study and 4.4% in the second study (Henriques et al. 2011; Tolf et al. 2012). Torrontegi et al. (2019) also found a prevalence of 0.3% and 6% and at least 44% of positive samples belonged to *A. platyrynchos*. These results support the idea that dabbling ducks (Anseriform order), and specially *A. platyrynchos*, are important IAV reservoirs, and when studies, such as ours, test less susceptible orders and species of birds the number of infected birds is brought inevitably down (Tolf et al. 2012). Another possibility is that circulating subtypes of IAV and respective prevalence differ between years, and the prevalence of IAV in the years that our samples were collected could have been low (Bárbara et al. 2017; Torrontegi et al. 2019). The differences in prevalence might also be related to natural temporal variation at different sampling locations, seasonal variation due to the presence of immunologically naïve birds, which can occur during breeding season and autumn migration, geographical location, circulating sub genotypes and interannual fluctuations in IAV prevalence (Tolf et al. 2012; Torrontegi et al 2019). Some authors have also suggested that southern latitude birds have less prevalence of IAV than birds from northern latitudes (Wallesten et al. 2007; Busquets et al. 2010). More longitudinal studies focused on waterfowl communities would be needed to understand our results and give an insight into the validity of these assumptions.

USUV, closely related to WNV, was a rather inconspicuous virus, and until some years ago, only a few studied it. But, in the last 20 years, USUV was demonstrated to circulate in several European countries, been associated with mortality events in birds and even caused disease in humans, which caught the attention of the scientific community (Vilibic-Cavlek et al. 2020). In Portugal there are no documented cases of USUV circulation, however in Spain at least two reported cases of circulation in birds exist. The first one corresponds to a flavivirus surveillance study carried out between 2011 and 2012, in southern Spain, which reported a 10% prevalence of USUV neutralizing antibodies in hunted red-legged partridges (*Alectoris rufa*) and common pheasants (*Phasianus colchicus*) (Llorente et al. 2013). The second one reported the isolation of USUV in two *T. philomelos* caught at a mortality event of approximately 10 birds in southern Spain in 2012 (Höfle et al. 2013).

In our study all samples were negative for the presence of USUV RNA, and various hypothesis could justify this negative result. First, and considering that if the virus circulates in Spain, it is possible that it also circulates in Portugal, it would be more likely to assert whether the virus circulates in our country through testing for the presence of USUV neutralizing

antibodies. If that is the case, and the virus circulates in Portugal, the absence of positive RNA samples may be due to low sample size and/or due to a low viraemia phase, as occurs with WNV. Since most documented cases of symptomatic infection in Europe have occurred in birds of this genus, namely *T. merula* and *T. philomelos*, one can also hypothesise that certain species of birds are more susceptible to USUV, such as birds from the *Turdus* genus, and more representation from these species would be needed in our study (Weissenböck et al. 2002; Chvala et al. 2007; Höfle et al. 2013). In our study only two birds of these genus were sampled, from the *T. merula* species; if more individuals of these genus were sampled, maybe the probability of detecting USUV would increase. Another possibility is that the circulation of virus in our country is so low that, in order to detect it, the sample size would need to be much larger. More studies would be needed to test each one of these hypotheses.

Despite absence of proof of USUV circulation in Portugal, it remains a high-risk country due to the millions of migrating birds that cross its territory and due to the circulation of the virus in Spain. It is of great concern that these birds, such as northern migrants coming from countries where USUV circulates in birds, especially from the *Turdus* genus such as redwing (*Turdus iliacus*), fieldfare (*Turdus pilaris*), *T. philomelos* and ring ouzel (*Turdus torquatus*) may introduce the virus into Portugal (Essbauer et al. 2013).

Contrary to USUV, WNV is a well-studied virus, especially in North America and Europe since veterinarians started to notice the death of multiple birds in New York City in 1999. In Europe, it has been described since the 1960s (Formosinho et al. 2006). In Portugal, the virus was first described and isolated from mosquitoes in 1971 (Barros et al. 2011) and, since then, research teams in Portugal have been studying the virus and testing for its presence in mosquitoes, horses, and birds. However, information regarding WNV prevalence in wild bird populations of Portugal is scarce. Formosinho et al. (2006) conducted a survey from 1999 to 2002 and found flavivirus neutralizing antibodies in 11.9% (16/134) of the sample and, later, Barros et al. (2011) conducted a survey between 2004 and 2010 in which a WNV neutralizing antibody prevalence of 19.80% (23/116) in wild birds' species was reported. To the authors' knowledge, those were the only survey studies of WNV made in wild bird populations of Portugal. The prevalence of WNV neutralizing antibodies found in our study was 13.7% (10/73), which is very similar to the prevalence found by Formosinho et al. (2006) but a lower than the prevalence found by Barros et al. (2011).

The differences found between the work of Barros et al. (2011) and our study may be simply due to a low sample number of birds tested for the presence of WNV neutralizing antibodies in our work or due to the sampled species being very different in both studies. While in our study only native species were tested, in the study by Barros et al. (2011) a majority of

the sample corresponded to zoological gardens (zoo) birds [55.17% of sampled birds (64/116)], which represent species that are not native to our ecosystems and, thus, can potentially be more susceptible to WNV. Other multiple factors associated with the substantial proportion of zoo birds within the sample may account for the differing prevalence between studies including: a) species size, most zoo bird species tend to be medium to large birds, and according to some authors, large species may be more prone to be bitten by vectors due to the amount of CO_2 they release (Burkett-Cadena et al. 2014; Llopis et al. 2016); b) presence of ponds in zoo enclosures, which, can be ideal habitats for mosquitoes breeding, while native species can live relatively far from water courses; c) higher density of animals compared to what occurs in nature, increasing the probability of a mosquito biting infected hosts and immunologically naïve birds; d) it is harder for animals to find shelter from vectors and animals spend, inevitably, less time flying, giving more opportunities for vectors to bite them; e) transference of animals between zoos due to participation in breeding programs or other reasons, with some of these animals possibly being infected and transporting the virus to new areas or vice-versa. In our neighbouring country, Spain, prevalence of neutralizing antibodies in wild birds has been reported ranging from 2.2% to 10.4% (Figuerola et al. 2007; Figuerola et al. 2008; López et al. 2008; López et al. 2011). Various factors could contribute to the discrepancy between these studies and ours, such as the sampled species, age, geography, habitat, ecology, season, and year. As such, the comparison between this study and others should be made with caution.

Six out of the 23 species (26.09%) sampled in our study had WNV neutralizing antibodies, namely *A. monachus, A. nisus, B. buteo, C. gallicus, H. pennatus* and *C. ciconia*. These species have different ecological and behavioural traits, and it is difficult to associate some of these traits with WNV infection (Equipa Atlas 2018). However, the statistical analysis allowed for the identification of relevant associations between some general characteristics, such as between bird order and the positive outcome in WNV serological test. Birds belonging to the Accipitriformes order had 35.85 more odds of having a positive WNV serological test result than other species [OR = 35.85, CI 95%: 4.76-840.82; ($p<0.001$)] contrary to birds belonging to the Strigiformes order, which were more frequently associated with a “negative WNV serological test result” [OR = 0, CI 95%: 0-0.76; ($p<0.05$)]. Other orders had no statistically relevant association with outcome in WNV serological test. Species’ biological and ecological factors, such as habitat preference, period of activity and proximity to other individuals of the same or different species, influence its contact with vectors and therefore, may make them more prone to infection than other species. The association between Accipitriformes and a positive outcome can be explained in ecological terms. European Accipitriformes are diurnal birds of prey, meaning their lowest activity period is during the night,

when foraging mosquitoes typically feed (Anderson et al. 2007; Burkett-Cadena et al. 2014). As birds of prey, they can feed on other smaller birds that may be infected with WNV, and thus become infected themselves (Chancey et al. 2015). As said earlier, *Cx. pipiens* does not randomly feed on bird species according to their abundance and has a marked preference for feeding from large raptors because they exhale larger amounts of CO_2, and, since the smallest Accipitriform species in our study, *A. nisus*, weights at least 100gr and all other Accipitriform sampled species weight at least 500gr, one can hypothesise that this is a big factor in Accipitriformes being associated with a positive WNV serological result (Burkett-Cadena et al. 2014; Llopis et al. 2016). One study made in Spain contradicts our study and refers that the association between bird order and the presence of WNV neutralizing antibodies was not significant (Figuerola et al. 2008). However, Figuerola et al, 2008, did not analyse the Accipitriformes order, raising the possibility of a different conclusion if the order was included. A macroecology study made by Tolsá et al. (2018) referred that Accipitriformes are potential reservoirs of WNV, corroborating our hypothesis. However, to the authors' knowledge, no experimental inoculation studies were performed in birds of this order, and therefore the possibility of transmissions to other species remains undetermined (Tolsá et al. 2018).

Animal species were also tested separately to assess whether each species was significantly associated with each outcome. *B. buteo* [OR = 9.31, CI 95%: 1.80-49.78; ($p<0.05$)] and *H. pennatus* [OR = Inf, CI 95%: 1.92-Inf; ($p<0.05$)] were significantly associated with the outcomes, having a more frequent "positive WNV serological test result". However, these associations should be taken with caution, since they are retrieved from a very small sample and the results may be biased. The factors that could have contributed to *B. buteo* and *H. pennatus* being significantly associated with having a more frequent "positive WNV serological test result" are the same as the factors that can explain Accipitriformes also having this statistically significant association: both are diurnal birds, both consume small birds, thus release more CO_2 predisposing them to mosquitoes' bites (Burkett-Cadena et al. 2014; Llopis et al. 2016).

In our study Strigiformes were associated with having a negative serological WNV test [OR = 0, CI 95%: 0-0.76; ($p<0.01$)]. In Europe, to the author's knowledge, seven studies have sampled *S. aluco* specimens, the most frequent Strigiform sampled in our study. A total of 55 *S. aluco* were sampled, with only two individuals being positive (3.64%) (Linke et al. 2007; Balança et al. 2009; López et al. 2011; Ziegler et al. 2012; Burkett-Cadena et al. 2014; Llopis et al. 2015; Jurado-Tarifa et al. 2016; Michel et al. 2019; Tolsá et al. 2018). This could mean that *S. aluco*, and potentially other European Strigiform species may be more prone to having a negative serological WNV test. Various factors could contribute to this. First, owls are nocturnal birds and, thus, are normally flying and more active during the night, making them

less available to foraging mosquitoes, that feed normally during the night (Anderson et al. 2007; Burkett-Cadena et al. 2014). Secondly, although all sampled Strigiform species sampled can feed on birds, they feed preferably on, depending on the species, small rodents, lagomorphs, or insects, and, thus, have less probability of being infected by the oral route than species that feed commonly on birds (STRI 2020). Other species-specific parameters, including body heat, host defence mechanisms, flight behaviour and host tolerance to mosquitoes could also have played a role in the Strigiformes being more prone to having a negative serological WNV test in our study (Edman et al. 1974; Osório et al. 2010; Takken et al. 2013).

The association between sex and positive outcome in serological test was also statistically significant, with males having 14 more odds of having a positive result than females [OR = 14, CI 95%: 1.14-480.95; ($p<0.05$)]. This is probably a type I error due to low sample size, since unknown sex bird were excluded from the data in this case and only 15 birds entered the test. Moreover, sex identification *in vivo* in some bird species is phenotypically very hard and even impossible in some cases, so one must bear in mind that it is a possibility that errors in sex identification in some species through phenotype may occur. Studies made in American Crow (*Corvus brachyrhynchos*) have not found sex to be a risk factor in WNV infection (Ludwig et al. 2002; Yaremych et al. 2004) and, to the authors' knowledge, the association between sex and WNV infection was never documented, so this result must be viewed with caution.

The association between age and positive outcome in WNV serological test was not statistically significant [OR = 0, IC 95%: 0-1.09; ($p=0.05$)]. While some studies also corroborate our findings and found no association between age and presence of WNV neutralization antibodies others suggest the contrary, that adults are, indeed, more prone to having WNV neutralizing antibodies (Ludwig et al. 2002; (Ringia et al. 2002; Figuerola et al. 2007; Komar et al. 2013). These differences between studies may be due to the season when birds were sampled, heterogenicity of the sample regarding species, size and habitat, and natural fluctuation of the virus prevalence between years, so assumptions regarding how different ages can influence risk and WNV prevalence, so this result should be taken with caution.

In our study, the association between migratory status and outcome in serological test was not statistically significant [OR = 0.65, CI 95%: 0.14-2.73; ($p=0.72$)]. Although some authors have found an association between localization of WNV outbreaks and migratory flyways in America, which point to the possibility of migratory birds dispersing the virus, to the authors' knowledge there are no reports of testing the association between a bird's migratory status and outcome in WNV serological test (Reed et al. 2003: Swetnam et al. 2018). This could mean the migrant and non-migrant species that occur in Portugal are equally prone to

be infected with WNV. This can corroborate the idea that WNV is endemic to continental Portugal and the African countries to which birds migrate. However, in our study individuals of resident species were overrepresented, which could have influenced the result. Moreover, other characteristics such as sample size, species represented, number of individuals representing each species or season when the birds were sampled could have influenced these results, so more studies to corroborate our hypothesis will be needed. Moreover, if future studies find an association between migratory species and outcome in WNV serological test, one must bear in mind that finding WNV neutralization antibodies or even isolating WNV from migrating birds does not necessarily establish them as WNV dispersion factors.

Although no association of migratory status and having a positive outcome in WNV serological test was found, knowledge of bird species' migratory behaviours can be important as it allows us to gather some epidemiological data of the WNV infection of the positive birds from our study. First, *C. ciconia* (sample 327-LxCRAS), *H. pennatus* (samples 243-CRAS-HVUTAD and 244-CRAS-HVUTAD) *C. gallicus* (sample 209-CRAS-HVUTAD) are migrant species so their contact with WNV may have occurred in either continental Portugal or Africa. Certain *C. ciconia* populations are resident and do not migrate to Africa, so it is a possibility that this individual has never migrated and, indeed, contacted with WNV in Portugal (Gilbert et al. 2016). *A. monachus* (sample 237-CRAS-HVUTAD) and *A. nisus* (sample 210-CRAS-HVUTAD) are resident species, so it is highly likely that they contacted with WNV in Portugal. It is also possible, in the case of *A. monachus, A. nisus* and in the three *B. buteo* (samples 213-CRAS-HVUTAD, 214-CRAS-HVUTAD and 232-CRAS-HVUTAD) that were found in Castelo Branco and Vila Real, that they had contact with WNV in Spain, near the border with Portugal. The *B. buteo* (sample 328-LxCRAS) that was found in Sintra, almost certainly contacted with WNV in Portugal since this species' territories tend to be small and dispersion movements tend to be short (Schindler et al. 2012).

Association between region of the country and outcome in serological test was not statistically significant (p=0.23). However, considerations regarding this should be made with caution, since 92.31% (24/26) of the samples from the South region (comprising Lisbon Metropolitan area, Alentejo and Algarve) are from the Lisbon Metropolitan area, and only one sample from Algarve and two from Alentejo were collected, so there is little representativity of the South region in this work, which, consequently, could have influenced the results. Alentejo and Algarve have the ideal characteristics for the life cycle of the vectors due to its warmer temperature and the presence of bodies of water (Freitas et al. 2012). Moreover, all the known cases of WNV infection in humans and horses and most of the known serological positive results in birds were from individuals that had been in or lived in Algarve or Alentejo (Barros et al. 2017). So, it is possible that due to the low sampling of birds from this region, the prevalence

of WNV neutralization antibodies in the south was underestimated. It is also possible that the prevalence is identical in the entire country and a survey bias in the past has led to this misconception.

An association between cause of admission and WNV was also not found (p= 0.08) which is expected, since the presence of antibodies does not indicate active infection but only contact with the virus and, thus, there are no motives for a special cause of admission to be significantly associated with it.

Our study did not find WNV RT-PCR positive samples in wild birds. Although WNV has been detected from mosquitoes and humans in Portugal, it has never been detected from birds in Portugal. Barros et al. (2011) tested 860 bird samples for WNV RNA and all those samples were negative. In Spain, WNV lineage 1 RNA has been detected in two golden eagles (*Aquila chrysaetos*) and one Bonelli's eagle (*Hieraaetus fasciatus*) (López and Jiménez-clavero 2008). Moreover, another Spanish study found viral RNA in 80% of Spanish imperial eagle (*Aquila adalberti*) specimens sampled (8/10). These included clinically healthy animals those with signals compatible with WNV infection that died of secondary infection (Höfle et al. 2008). In our study none of these species was analysed, because these are endangered or critically endangered species with low populations numbers and their admission in wildlife centres is rare (Ramos 2016). It is possible that the Portuguese population of these animals may also be infected with WNV, especially the *A. adalberti* population due to their habitat and behaviour, so future studies testing this hypothesis could be important for the management of the species in Portugal.

One study also documented a WNV genotype II RNA positive sample in two *A. gentilis* individuals in Catalonia in 2017. Evidence of westward spread of WNV lineage 2, a lineage that had been restricted to Central and Eastern area of Europe. Until now, that is the only documented case of WNV genotype II infection in the Catalonian region and in the Iberian Peninsula, but may indicate that in the next years, WNV lineage 2 can potentially circulate in the rest of Spain and in Portugal (Busquets et al. 2019).

The lack of positive RNA samples in our study could be, according to some authors, due to the fact that few European birds develop symptoms and most of them are able to quell the infection (Hubálek and Halouzka 1999; Gray and Webb 2014). Moreover, the viraemic phase usually lasts less than 7 days, hampering viral RNA detection (Komar et al. 2003; Figuerola et al. 2008).

5. LIMITATIONS OF THE STUDY AND FUTURES PERSPECTIVES

As shown before, interspecific variations can be caused by different susceptibility to WNV, WNV-vector biting preferences and differences in avian ecology which result to different exposure to vectors (Edman et al. 1974; Osório et al. 2010; Takken et al. 2013). The identification of each cause, responsible for these interspecific variations is difficult, especially in studies such as ours which sampled a large number of different species, from different geographical sites and habitats, different vector exposition and different risk factors (Figuerola et al. 2007; López et al. 2011). An alternative approach for a more robust risk assessment of infection would imply increasing the sample, with a higher representativeness for each district, so that future studies could assess the association between various regions of the country and habitats. Moreover, focusing the sampling effort in one sentinel species, similar to what occurs in North America, would also be a good alternative approach since comparing differences between regions, age, sex, season, and other biological differences in a unique species would also provide more robust results since behavioural and ecological variations would be reduced to only intraspecific variations. *B. buteo* is, in our opinion, one of the best species to use in this case, since it is a large diurnal raptor, which is associated with susceptibility to WNV infection and allows for easier collection of samples than smaller species; is frequently admitted in rehabilitation centres, and WNV neutralizing antibodies have been found in this species. Moreover, it is very common in Portugal, is present in the whole territory and does not migrate, thus providing more clues regarding the circulation of WNV in Portugal. The collected data would deliver a more comprehensive data for the evaluation of WNV infection risk not only for birds, but also for horses and humans as well and would allow the drawing of a national risk map.

In future studies, sampling more individuals from each species and from each order would also be interesting so a more robust statistical analysis of biological factors and susceptibility of different orders and species could be made.

Finally, it would also be interesting to sample more individuals of endangered Accipitriformes considered as most susceptible to WNV, such as *A. chrysaetos*, *A. adalberti* and *A. fasciatus*. Besides their higher susceptibility to WNV, being endangered species, it would be of interest to study the real impact of WNV in their population dynamics. Moreover, it would be important to sample other neighbouring species, to investigate the potential contact with the virus, assessing if it exists in these areas or if these species are in fact more prone to be bitten by the vector and more susceptible to the virus.

Regarding USUV in Portugal additional surveillance in the future is necessary. Considering the hypothesis that birds from the *Turdus* genus are more predisposed to USUV

infection it would be interesting to test not only *T. philomelos* specimens, which migrate from countries were USUV circulates, but also *T. merula*, a resident species to test the epidemiology in both migrant and non-migrant species. As said earlier, focusing on these species would also allow for a comparison of biological differences (i.e. age, sex, season) with more robust results since behavioural and ecological variations would be reduced to only intraspecific variations.

Wildlife rehabilitation centres are the ideal platform to collect samples for WNV and USUV surveillance since they receive a considerable number of specimens per year, tend to receive many diurnal raptors and birds from the *Turdus* genus and their professionals are accustomed to sample collection. Ringing activities would also be a good option, as they tend to cover most of the continental territory, enabling the collection of samples from the whole country, and the majority of them focus on ringing of small Passeriformes.

Regarding IAV, we suggest that sampling for future studies should focus more on Anatid species, mainly in *A. platyrynchos*, since it is referred that their IAV prevalence is higher than in other species (Wallensten et al. 2007; Jourdain et al. 2010). In accordance with the suggestions of Tolf et al. (2012) that IAV prevalence is highest during summer months in countries of southern Europe, we suggest that sampling in Portugal should be performed in this period. Again, focusing on one species would allow for more robust results regarding comparison of biological differences.

In the case of AOaV-1, it would be important to perform a full genome phylogenetic analysis to assure that the observed clusters are indeed the most similar to the strains we characterized or any other strain. It would be interesting to study if the strains found in *S. decaocto*, are antigenically characterized as PPMV-1 and would induce clinical signs and mortality in chickens or if, similarly to some PPMV-1, they do not cause clinical signs and mortality in chickens (Dortmans et al. 2009; Zhan et al. 2020). It would also be important to make histological, bacteriological, and mycological exams in the cadavers in order to understand if AOav-1 was the primary cause of death or if other infection or health condition caused their death.

We consider that additional studies should focus on the prevalence of AOaV-1 infection in wild Columbiformes and in the consequences for wild populations, poultry and even humans. Furthermore, it is important that public health doctors receive more information regarding the risk that AOaV-1 poses to humans and more studies should be made focusing on this subject.

Finally, it is important to refer that the rationale of this study may have influenced and biased the collected data. All samples were collected from wildlife rehabilitation centres, increasing the proportion of injured or sick animals compared to healthy animals, higher than it would be in a natural setting, potentially increasing the prevalence of some diseases. Sample

collection from rehabilitation centres also influences the sampled specie, since some species with a low representation in the wild populations are highly represented in rehabilitation centres and vice-versa, potentially leading to overestimation and underestimation of disease prevalence.

Still regarding the collection of samples in live animals, one must remember that the primary goal of a rehabilitation centre is to release the animal back into the wild and thus, in certain cases, the collection of samples is not recommended, thus biasing the study even more. Wildlife rehabilitation centres also tend to receive more animals from nearby districts and, since wildlife rehabilitation centres are not evenly distributed in the Portuguese territory, we could not collect samples from every district, inducing an under representation of some districts in our study. Although it is hard to conduct unbiased studies in wild animal populations, further studies should try to address these problems. The collection of a larger sample, for a longer period of time, with more sampled species involving all wildlife rehabilitation centres from Portugal as well as other activities where samples collection is possible - such as ringing sessions - would probably address some of these problems and deliver more robust data.

6. CONCLUSION

In conclusion this study was focused on survey for evidence that WNV, USUV, IAV and AOaV-1 circulate in wild birds in Portugal and to associate these pathogenic agents with specific biological factors such as geographical location, bird order and species, age, sex and migratory status. Circulation of AOaV-1 and WNV was confirmed while no evidence of USUV and IAV circulation was found.

Accipitriformes, *B. buteo*, *H. pennatus* and male birds appear to be associated with a positive WNV serological result, while Strigiformes appear to be associated with a negative WNV serological result. *S. decaocto* appear to be associated with a positive AOaV-1 test result.

This study revealed the importance of doing more research regarding AOaV-1 infections in wild birds because of their potential for crossing the wild-domestic animals' barrier, their zoonotic potential, and the possible impacts on endangered species conservation. More studies regarding the impact of this virus in endangered species, especially of the Columbidae family, are also needed.

Samples collected from two S. decaocto had a R-R-Q-K-R*F aminoacidic sequence in the fusion protein cleavage site and could potentially be highly virulent for chickens. Phylogenetic analysis confirmed that these sequences clustered with other AOaV-1 sequences from genotype XXI, subgenotype XXI.2, mainly with pigeons and other dove species.

It is also important to continue to survey the presence of WNV, USUV and IAV in our country, actively and passively. Knowing the birds' biological and ecological factors is essential to evaluate the association between these factors and the presence of infectious diseases, to possibly corroborate or oppose the results found in this work and other studies regarding our country. Wildlife rehabilitations centres across Portugal are an ideal platform to study the pathophysiology and epidemiology of these viruses in endemic species since together they receive thousands of wild birds every year. It is also relatively easy to implement a surveillance network in collaboration with research laboratories.

Considering the potential for human, domestic, and wild animal risks that these four viruses have, it is critical to continue investigating their epidemiology to prevent or mitigate future outbreaks that can eventually occur.

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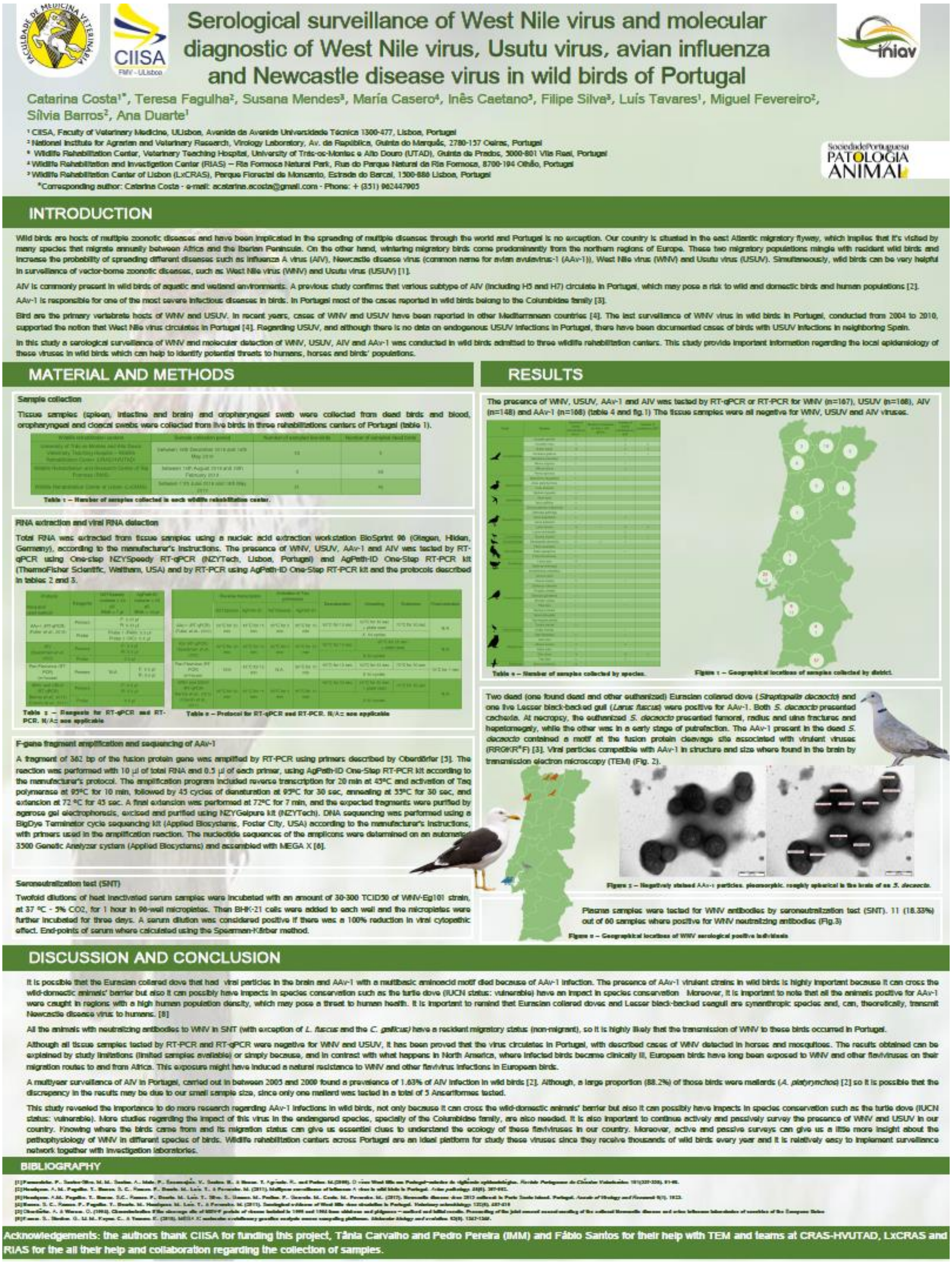
8. APPENDIX

Appendix 1 - Poster presented in the XXIV Encontro da Sociedade Portuguesa de Patologia Animal (June 15th-16th, 2019, Vila Real, Portugal).



Appendix 2 - Poster presented in presented in the Wildlife & Game Management Innovation Summit (June 28th - 29th 2019, Lisbon, Portugal).

NEWCASTLE VIRUS DISEASE (NDV) IN TWO EURASIAN COLLARED DOVES (*Streptopelia decaocto*): A THREAT TO EUROPEAN TURTLE DOVE (*Streptopelia turtur*) POPULATIONS?

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INTRODUCTION

Avian avulavirus-1 (AAv-1), commonly known as Newcastle disease virus, is responsible for one of the most severe infectious diseases in birds. In Portugal most of the cases reported in wild birds belong to the Columbidae family, mainly in rock pigeons (*Columba livia*) but also in European turtle doves (*Streptopelia turtur*) (in Porto Santo) [1]. Moreover, it is important to note that AAv-1 may be deadly to members of the Columbidae family.
Eurasian collared dove (*Streptopelia decaocto*) is a closely related species to *S. turtur*, a cinegetic species considered vulnerable [2] whose number of breeding individuals had a decline of approximately 80% in Portugal in the last years [3].
In this study a molecular detection of AAv-1 was conducted in *S. decaocto* admitted to three wildlife rehabilitation centers.
This study provide important information regarding the local epidemiology of AAv-1 and can help to identify potential threats to other wild birds, such as *S. turtur*.

MATERIAL AND METHODS

Sample collection (Table 1)
Dead birds: Tissue samples (spleen, intestine, brain) and oropharyngeal swab
Live birds: oropharyngeal and cloacal swabs.

Wildlife Rehabilitation center	Time of death	Time of sample collection	Dead animals	Live animals
University of Trás-os-Montes and Alto Douro Veterinary Teaching Hospital – Wildlife Rehabilitation center (CRAS-HVUTAD)	N/A	1st of May 2019	0	1
Wildlife Rehabilitation and Research center of Ria Formosa (RIAS)	23th of March to 20th August 2018	2nd of February 2019	6	0
Wildlife Rehabilitation center of Lisbon (LxCRAS)	9th of June 2018	13th of June 2018	1	0

Table1 - Number of samples collected in each rehabilitation center.

RNA extraction and viral RNA detection
Total RNA extraction: workstation BioSprint 96 (Qiagen, Hilden, Germany)
The presence of AAv-1 was tested by RT-qPCR using One-step NZYSpeedy RT-qPCR (NZYTech, Lisboa, Portugal) and by RT-qPCR using AgPath-ID One-Step RT-PCR kit (ThermoFisher Scientific, Waltham, USA) and the protocols described in Tables 2 and 3.

Used method	Reagents	NZYSpeedy (reaction volume = 20 µl)	AgPath-ID (reaction volume = 25 µl)
RT-qPCR Fuller et al., 2013	RNA	7 µl	10 µl
	Primers	F: 0.25 µl R: 0.25 µl	
	Probe	Probe 1 (FAM): 0.5 µl Probe 2 (VIC): 0.5 µl	

Table2 - Reagents for RTqPCR and RT- PCR.

	Reverse transcription	Activation of Taq polimerase	Denaturation	Annealing	Extension	Final extension
RT-qPCR Oberdorfer & Werner, 1994	45° for 20 min	95° for 10 min.	95° for 30 sec	55° for 30 sec	72° for 45 sec	72° for 7 min

Table4 - Protocol for RT-PCR.

F-gene fragment amplification and sequencing of AAv-1
A fragment of 362 bp of the fusion protein gene was amplified and sequenced using the protocol described in Figure 1.

Figure 1 - Protocol for F-gene fragment amplification and sequencing of AAv-1.

RESULTS

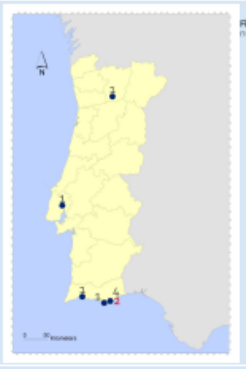

Figure2 - Geographical location where the sampled *S. decaocto* where found. Black numbers= number of samples collected; red numbers= number of positive samples

Figure3 - *S. decaocto*.

Two dead (one found dead and other euthanized) *S. decaocto* (Fig. 3) were positive for AAv-1.
The AAv-1 present in the dead *S. decaocto* contained a motif at the fusion protein cleavage site associated with virulent viruses (RRQKR*F) [7].
Viral particles compatible with AAv-1 in structure and size where found in the brain by transmission electron microscopy (TEM) (Fig. 2).

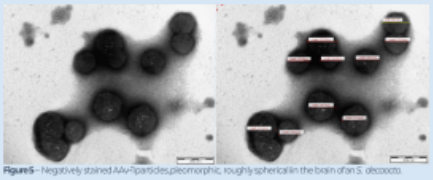

Figure5 - Negatively stained AAv-1 particles, pleomorphic, roughly spherical in the brain of an *S. decaocto*.

DISCUSSION AND CONCLUSION

Figure4 - *S. turtur*

It is possible that the Eurasian collared dove that had viral particles in the brain and AAv-1 with a multibasic aminoacid motif died because of AAv-1 infection. The presence of AAv-1 virulent strains *S. decaocto* in Portugal can pose a threat to endangered species, such as *S. turtur* (Fig. 4), because these two species can sometimes share habitats with one another. Although, it is crucial to note that we could not sample *S. turtur* specimens and it is possible that the virus already circulates in this species in continental Portugal.
It is also important to note that AAv-1 has zoonotic potential and caution should be taken by hunters when handling these animals [8].
We consider that further research should be made to study the prevalence of AAv-1 in wild Columbiformes, its pathogenicity to these species and role of *S. decaocto* in the ecology of AAv-1. More studies regarding the impact of this virus in *S. turtur* are also needed.
Considering the risks that this disease can have in population numbers of *S. turtur* together with other possible factors (habitat loss/modification, illegal hunting, climate change, etc.) that may be contributing to populations decline, we suggest that, better game management (e.g. reduction of bag limit) together with other conservation measures (protection of most important habitats) should be considered. Moreover, considering the lack of information regarding this subject, to the authors knowledge and according to the precautionary principle, a temporary ban on *S. turtur* hunting should also be highly considered [9].
Wildlife rehabilitation centers across Portugal are an ideal platform for study these viruses since they receive thousands of wild birds every year and it is relatively easy to implement surveillance network together with investigation laboratories.

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Appendix 3 – Sampled individuals' information, RT-qPCR results and WNV seroneutralization results. Species marked with an asterisk (*) are exotic species.

Centre	Sample	Sample collection day	Locality where was found	Region	Status	Order	Species	Migratory status	Age	Sex	Admission cause	RT-qPCR				WNV Seroneutralization
												WNV	USUV	AOaV-1	IAV	
LxCRAS	1	11-06-2018	Lisboa	South	D	Passeriformes	*G. glandarius*	R	JUV	M	Nestling	N	N	N	N	NA
LxCRAS	2	11-06-2018	Lisboa	South	D	Strigiformes	*A. otus*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	3	11-06-2018	NA	NA	D	Gruiformes	*G. chloropus*	R	JUV	M	Nestling	N	N	N	N	NA
LxCRAS	4	11-06-2018	Lisboa	South	D	Apodiformes	*A. apus*	M	AD	M	Unknown	N	N	N	N	NA
LxCRAS	5	13-06-2018	Paço de Arcos	South	D	Falconiformes	*F. tinnunculus*	R	JUV	M	Nestling	N	N	N	N	NA
LxCRAS	6	13-06-2018	Arruda dos Vinhos	South	D	Columbiformes	*S. decaocto*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	7	18-06-2018	Loures	South	D	Strigiformes	*A. noctua*	R	JUV	M	Nestling	N	N	N	N	NA
LxCRAS	8	18-06-2018	NA	NA	D	Apodiformes	*A. pallidus*	M	AD	F	Trauma	N	N	N	N	NA
LxCRAS	9	18-06-2018	Cascais	South	D	Apodiformes	*A. pallidus*	M	AD	M	Trauma	N	N	N	N	NA
LxCRAS	10	18-06-2018	Lisboa	South	D	Apodiformes	*A. pallidus*	M	AD	F	Trauma	N	N	N	N	NA
LxCRAS	11	22-06-2018	Barreiro	South	D	Passeriformes	*C. corone*	R	JUV	M	Trauma	N	N	N	N	NA
LxCRAS	12	22-06-2018	Sintra	South	D	Accipitriformes	*B. buteo*	R	AD	M	Trauma	N	N	N	N	NA
LxCRAS	13	27-06-2018	Samora Correia	South	D	Accipitriformes	*P. apivorus*	M	AD	M	Trauma	N	N	N	N	NA
LxCRAS	14	06-07-2018	NA	NA	D	Falconiformes	*F. tinnunculus*	R	JUV	M	Trauma	N	N	N	N	NA
LxCRAS	16	09-07-2018	Pinhal Novo	South	D	Ciconiiformes	*C. ciconia*	M	JUV	F	Trauma	N	N	N	N	NA
LxCRAS	17	09-07-2018	Lisboa	South	D	Falconiformes	*F. tinnunculus*	R	AD	F	Unknown	N	N	N	N	NA
LxCRAS	18	14-07-2018	Barreiro	South	D	Accipitriformes	*B. buteo*	R	AD	NA	Unknown	N	N	N	N	NA
LxCRAS	19	14-07-2018	Almada	South	D	Passeriformes	*G. glandarius*	R	JUV	M	Trauma	N	N	N	N	NA
LxCRAS	20	18-11-2018	Olivais	South	D	Charadriiformes	*L. michahellis*	R	JUV	M	Trauma	N	N	N	N	NA
LxCRAS	21	18-11-2018	Lisboa	South	D	Apodiformes	*A. pallidus*	M	JUV	NA	Unknown	N	N	N	N	NA
LxCRAS	22	18-11-2018	Sintra	South	D	Strigiformes	*A. noctua*	R	AD	M	Trauma	N	N	N	N	NA
LxCRAS	23	18-11-2018	Almada	South	D	Passeriformes	*C. corone*	R	AD	M	Trauma	N	N	N	N	NA
LxCRAS	24	18-11-2018	Sintra	South	D	Passeriformes	*E. rubecula*	M	AD	NA	Trauma	N	N	N	N	NA
LxCRAS	25	18-11-2018	Barreiro	South	D	Passeriformes	*S. unicolor*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	26	18-11-2018	Linda-a-velha	South	D	Passeriformes	*T. merula*	R	JUV	F	Unknown	N	N	N	N	NA
LxCRAS	27	18-11-2018	Lisboa	South	D	Apodiformes	*A. apus*	M	JUV	M	Unknown	N	N	N	N	NA
LxCRAS	28	18-11-2018	Lisboa	South	D	Charadriiformes	*L. michahellis*	R	AD	F	Paretic syndrome	N	N	N	N	NA
LxCRAS	29	18-11-2018	NA	NA	D	Passeriformes	*T. guttata**	R	AD	M	Unknown	N	N	N	N	NA
LxCRAS	30	18-11-2018	V.F.Xira	South	D	Accipitriformes	*B. buteo*	R	JUV	NA	Trauma	N	N	N	N	NA
LxCRAS	31	18-11-2018	V.F.Xira	South	D	Charadriiformes	*L. fuscus*	M	AD	M	Paretic syndrome	N	N	N	N	NA
LxCRAS	32	18-11-2018	Almada	South	D	Passeriformes	*A. cristatellus**	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	33	18-11-2018	V.F.Xira	South	D	Strigiformes	*B. bubo*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	34	18-11-2018	Caneças	South	D	Passeriformes	*T. merula*	R	AD	M	Trauma	N	N	N	N	NA
LxCRAS	35	18-11-2018	Lisboa	South	D	Passeriformes	*F. coelebs*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	36	18-11-2018	NA	NA	D	Passeriformes	*S. atricappila*	R	AD	M	Trauma	N	N	N	N	NA
LxCRAS	37	18-11-2018	Barreiro	South	D	Passeriformes	*H. rustica*	R	AD	F	Illegal captivity	N	N	N	N	NA
RIAS	38	15-08-2018	Portimão	South	D	Charadriiformes	*L. michahellis*	M	AD	F	Unknown	N	N	N	N	NA
RIAS	39	15-08-2018	Olhão	South	D	Charadriiformes	*L. audouinii*	R	JUV	M	Paretic syndrome	N	N	N	N	NA
RIAS	40	15-08-2018	Portimão	South	D	Charadriiformes	*L. michahellis*	R	AD	F	Paretic syndrome	N	N	N	N	NA

"A" – Alive; "AD" – Adult; "D" – Dead; "JUV" – Juvenile; "M" – Migrant; "N" – Negative; "NA" – Non-Available; "P" – Positive; "R" – Resident

Appendix 3 – Continuation.

Centre	Sample	Sample collection day	Locality where was found	Region	Status	Order	Species	Migratory status	Age	Sex	Admission cause	RT-qPCR				WNV Seroneutralization
												WNV	USUV	AOaV-1	IAV	
RIAS	41	15-08-2018	Portimão	South	D	Charadriiformes	*L. michahellis*	R	AD	M	Trauma	N	N	N	N	NA
RIAS	42	15-08-2018	NA	NA	D	Charadriiformes	*L. michahellis*	R	AD	F	Trauma	N	N	N	N	NA
RIAS	43	15-08-2018	Quarteira	South	D	Charadriiformes	*L. fuscus*	M	AD	F	Paretic syndrome	N	N	N	N	NA
RIAS	44	15-08-2018	Mértola	South	D	Ciconiiformes	*C. ciconia*	M	JUV	M	Trauma	N	N	N	N	NA
RIAS	45	18-08-2018	Alcantarilha	South	D	Strigiformes	*A. noctua*	R	AD	F	Trauma	N	N	N	N	NA
RIAS	46	18-08-2018	Olhão	South	D	Strigiformes	*A. noctua*	R	AD	F	Weakness	N	N	N	N	NA
RIAS	47	18-08-2018	Albufeira	South	D	Falconiformes	*F. tinnunculus*	R	JUV	F	Nestling	N	N	N	N	NA
RIAS	48	18-08-2018	Castro Verde	South	D	Falconiformes	*F. naumanni*	M	JUV	M	Trauma	N	N	N	N	NA
RIAS	49	18-08-2018	Tavira	South	D	Strigiformes	*T. alba*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	50	18-08-2018	Loulé	South	D	Strigiformes	*T. alba*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	51	18-08-2018	S. brás de Alportel	South	D	Strigiformes	*S. aluco*	R	JUV	F	Trauma	N	N	N	N	NA
RIAS	52	18-08-2018	Cerro S. Miguel	South	D	Strigiformes	*S. aluco*	R	AD	M	Trauma	N	N	N	N	NA
RIAS	53	18-08-2018	Tavira	South	D	Accipitriformes	*B. buteo*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	55	18-08-2018	Mértola	South	D	Accipitriformes	*B. buteo*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	56	18-08-2018	Pias	South	D	Accipitriformes	*B. buteo*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	57	18-08-2018	Moura	South	L	Accipitriformes	*A. pennata*	M	JUV	F	Weakness	N	N	N	N	NA
RIAS	58	29-08-2018	Vilamoura	South	D	Anseriformes	*S. clypeata*	M	AD	M	Suspicion of intoxication	N	N	N	N	NA
RIAS	59	29-08-2018	Vilamoura	South	D	Anseriformes	*M. strepera*	M	AD	M	Suspicion of intoxication	N	N	N	N	NA
RIAS	60	29-08-2018	Vilamoura	South	D	Gruiformes	*F. atra*	M	AD	M	Suspicion of intoxication	N	N	N	N	NA
RIAS	61	31-08-2018	Vilamoura	South	D	Gruiformes	*F. atra*	M	AD	M	Suspicion of intoxication	N	N	N	N	NA
RIAS	62	01-09-2018	Portimão	South	D	Strigiformes	*S. aluco*	R	AD	F	Trauma	N	N	N	N	NA
RIAS	63	01-09-2018	Vilamoura	South	D	Anseriformes	*A. clypeata*	M	AD	F	Suspicion of intoxication	N	N	N	N	NA
RIAS	64	15-08-2018	Carvoeiro	South	D	Charadriiformes	*L. fuscus*	M	AD	M	Paretic syndrome	N	N	N	N	NA
RIAS	100	30-09-2018	Beja	South	A	Accipitriformes	*A. pennata*	M	AD	NA	Unknown	N	N	N	N	NA
RIAS	101	NA	V. N. Milfontes	South	D	Suliformes	*M. bassanus*	M	JUV	M	Weakness	N	N	N	N	NA
RIAS	102	NA	Lagos	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Trauma	N	N	N	N	NA
RIAS	103	NA	Porto Covo	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Weakness	N	N	N	N	NA
RIAS	104	NA	Albufeira	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Unknown	N	N	N	N	NA
RIAS	105	NA	Tavira	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Weakness	N	N	N	N	NA
RIAS	106	NA	Castro Marim	South	D	Suliformes	*M. bassanus*	M	AD	NA	Trauma	N	N	N	N	NA
RIAS	107	NA	Tavira	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Weakness	N	N	N	N	NA
RIAS	108	NA	Castro Marim	South	D	Suliformes	*M. bassanus*	M	AD	NA	Weakness	N	N	N	N	NA
RIAS	109	NA	Carvoeiro	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Trauma	N	N	N	N	NA
RIAS	110	NA	Olhão	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Weakness	N	N	N	N	NA
RIAS	111	NA	Loulé	South	D	Suliformes	*M. bassanus*	M	AD	NA	Weakness	N	N	N	N	NA
RIAS	112	NA	VRSA	South	D	Suliformes	*M. bassanus*	M	JUV	NA	Weakness	N	N	N	N	NA
RIAS	113	20-11-2018	Beja	South	A	Strigiformes	*B. bubo*	R	AD	F	Trauma	N	N	N	N	NA
RIAS	114	06-12-2018	Faro	South	A	Passeriformes	*C. corax*	R	AD	NA	Illegal captivity	N	N	N	N	NA
RIAS	115	23-02-2018	Carvoeiro	South	D	Columbiformes	*S. decaocto*	R	NA	M	Unknown	N	N	N	N	NA
RIAS	116	23-02-2018	Olhão	South	D	Columbiformes	*S. decaocto*	R	AD	F	Weakness	N	N	N	N	NA

"A" – Alive; "AD" – Adult; "D" – Dead; "JUV" – Juvenile; "M" – Migrant; "N" – Negative; "NA" – Non-Available; "P" – Positive; "R" – Resident

Appendix 3 – Continuation.

Centre	Sample	Sample collection day	Locality where was found	Region	Status	Order	Species	Migratory status	Age	Sex	Admission cause	RT-qPCR				WNV Seroneutralization
												WNV	USUV	AOaV-1	IAV	
RIAS	117	23-02-2018	Faro	South	D	Columbiformes	*S. decaocto*	R	AD	F	Trauma	N	N	N	N	NA
RIAS	118	23-02-2018	Olhão	South	D	Columbiformes	*S. decaocto*	R	AD	M	Unknown	N	N	P	N	NA
RIAS	119	23-02-2018	Olhão	South	D	Columbiformes	*S. decaocto*	R	AD	M	Trauma	N	N	N	N	NA
RIAS	120	23-02-2018	Olhão	South	D	Columbiformes	*S. decaocto*	R	AD	M	Trauma	N	N	P	N	NA
RIAS	121	23-02-2018	Estói	South	D	Strigiformes	*A. noctua*	R	AD	NA	Trauma	N	N	N	N	NA
RIAS	122	23-02-2018	Portimão	South	D	Charadriiformes	*C. ridibundus*	M	AD	F	Paretic syndrome	N	N	N	N	NA
RIAS	123	23-02-2018	Carvoeiro	South	D	Charadriiformes	*C. ridibundus*	M	AD	M	Paretic syndrome	N	N	N	N	NA
RIAS	124	23-02-2018	Quarteira	South	D	Anseriformes	*A. clypeata*	M	AD	F	Suspicion of intoxication	N	N	N	N	NA
RIAS	125	23-02-2018	Quarteira	South	D	Anseriformes	*A. clypeata*	M	AD	F	Suspicion of intoxication	N	N	N	N	NA
RIAS	126	23-02-2018	Loulé	South	D	Accipitriformes	*B. buteo*	R	AD	F	Trauma	N	N	N	N	NA
RIAS	127	23-02-2018	Castro Marim	South	D	Accipitifromes	*M. migrans*	M	AD	M	Trauma	N	N	N	N	NA
RIAS	128	23-02-2018	Olhão	South	D	Strigiformes	*A. flammeus*	M	AD	F	Trauma	N	N	N	N	NA
RIAS	129	23-02-2018	Olhão	South	D	Strigiformes	*A. noctua*	R	JUV	F	Weakness	N	N	N	N	NA
RIAS	130	23-02-2018	Faro	South	D	Strigiformes	*A. noctua*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	131	23-02-2018	Olhão	South	D	Strigiformes	*A. noctua*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	132	23-02-2018	Castro Verde	South	D	Strigiformes	*A. noctua*	R	JUV	M	Trauma	N	N	N	N	NA
RIAS	133	23-02-2018	Olhão	South	D	Strigiformes	*A. noctua*	R	JUV	M	Unknown	N	N	N	N	NA
RIAS	134	23-02-2018	S. Brás Alportel	South	D	Strigiformes	*A. noctua*	R	AD	M	Illegal captivity	N	N	N	N	NA
RIAS	135	23-02-2018	Portimão	South	D	Strigiformes	*A. noctua*	R	JUV	M	Nestling	N	N	N	N	NA
RIAS	136	23-02-2018	Tavira	South	D	Strigiformes	*A. noctua*	R	AD	F	Nestling	N	N	N	N	NA
LxCRAS	137	11-11-2018	Oeiras	South	D	Accipitriformes	*B. buteo*	R	AD	NA	Trauma	N	N	N	N	NA
LxCRAS	138	11-11-2018	Setúbal	South	D	Falconiformes	*F. tinnunculus*	R	AD	M	Trauma	N	N	N	N	NA
LxCRAS	139	11-11-2018	Oeiras	South	D	Charadriiformes	*L. michahellis*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	140	11-11-2018	NA	NA	D	Charadriiformes	*L. michahellis*	R	AD	M	Unknown	N	N	N	N	NA
LxCRAS	141	11-11-2018	Palmela	South	D	Ciconiiformes	*C. ciconia*	M	AD	F	Trauma	N	N	N	N	NA
LxCRAS	142	11-11-2018	Setúbal	South	D	Charadriiformes	*L. michahellis*	R	JUV	F	Paretic syndrome	N	N	N	N	NA
LxCRAS	143	11-11-2018	Sintra	South	D	Charadriiiformes	*L. michahellis*	R	AD	F	Trauma	N	N	N	N	NA
LxCRAS	144	11-11-2018	Cascais	South	D	Suliformes	*M. bassanus*	M	AD	M	Unknown	N	N	N	N	NA
LxCRAS	150	11-11-2018	Ericeira	South	A	Suliformes	*M. bassanus*	M	JUV	NA	Paretic syndrome	N	N	N	N	NA
RIAS	151	23-02-2018	Quarteira	South	D	Gruiformes	*F. atra*	M	AD	F	Suspicion of intoxication	N	N	N	N	NA
RIAS	152	23-02-2018	Quarteira	South	D	Gruiformes	*F. atra*	M	AD	F	Suspicion of intoxication	N	N	N	N	NA
RIAS	153	NA	Almancil	South	D	Strigiformes	*A. noctua*	R	NA	NA	Trauma	N	N	N	N	NA
RIAS	154	NA	Portimão	South	D	Strigiformes	*A. noctua*	R	NA	NA	Trauma	N	N	N	N	NA
RIAS	155	NA	Albufeira	South	D	Suliformes	*M. bassanus*	M	NA	NA	Trauma	N	N	N	N	NA
CRAS-HVUTAD	200	18-12-2018	Lamego	Centre	A	Strigiformes	*A. noctua*	R	JUV	NA	Unknown	NA	NA	NA	NA	N
CRAS-HVUTAD	201	18-12-2018	Vila Real	North	A	Strigiformes	*A. noctua*	R	JUV	NA	Trauma	NA	NA	NA	NA	N
CRAS-HVUTAD	202	18-12-2018	Bragança	North	A	Accipitriformes	*M. milvus*	M	AD	NA	Trauma	NA	NA	NA	NA	N
CRAS-HVUTAD	205	26-12-2018	V. N. de Gaia	North	A	Accipitriformes	*A. gentilis*	R	JUV	F	Trauma	NA	NA	NA	NA	N
CRAS-HVUTAD	206	26-12-2018	Bragança	North	A	Accipitriformes	*M. milvus*	M	AD	NA	Trauma	NA	NA	NA	NA	N

"A" – Alive; "AD" – Adult; "D" – Dead; "JUV" – Juvenile; "M" – Migrant; "N" – Negative; "NA" – Non-Available; "P" – Positive; "R" – Resident

Appendix 3 – Continuation.

Centre	Sample	Sample collection day	Locality where was found	Region	Status	Order	Species	Migratory status	Age	Sex	Admission cause	RT-qPCR				WNV Seroneutralization
												WNV	USUV	AOaV-1	IAV	
CRAS-HVUTAD	207	26-12-2018	Poiares	North	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	NA	NA	NA	NA	N
CRAS-HVUTAD	208	28-12-2018	V. P. Aguiar	North	A	Pelecaniformes	*A. cinerea*	M	JUV	M	Trauma	NA	NA	NA	NA	N
CRAS-HVUTAD	209	28-12-2018	Canelas	North	A	Accipitriformes	*C. gallicus*	M	AD	NA	Trauma	N	N	N	N	P
CRAS-HVUTAD	210	09-01-2019	Boticas	North	A	Accipitriformes	*A. nisus*	R	AD	M	Trauma	N	N	N	N	P
CRAS-HVUTAD	211	11-01-2019	Vila Flor	North	A	Passeriformes	*C. corone*	R	AD	NA	Illegal captivity	NA	NA	NA	NA	N
CRAS-HVUTAD	212	23-01-2019	Gaia	North	A	Accipitriformes	*A. gentilis*	R	AD	F	Trauma	NA	NA	NA	NA	N
CRAS-HVUTAD	213	06-02-2019	Peso da Régua	North	A	Accipitriformes	*B. buteo*	R	AD	M	Trauma	N	N	N	N	P
CRAS-HVUTAD	214	06-02-2019	V. P. Aguiar	North	A	Accipitriformes	*B. buteo*	R	AD	F	Trauma	N	N	N	N	P
CRAS-HVUTAD	215	16-02-2019	Cinfães	Centre	A	Accipitriformes	*A. nisus*	R	AD	F	Trauma	N	N	N	N	N
CRAS-HVUTAD	216	16-02-2019	Peso da Régua	North	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	217	17-02-2019	Vila Flor	North	A	Accipitriformes	*B. buteo*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	218	17-02-2019	Gerês	North	A	Accipitriformes	*B. buteo*	R	AD	F	Illegal captivity	N	N	N	N	N
CRAS-HVUTAD	219	20-02-2019	Peso da Régua	North	A	Accipitriformes	*S. aluco*	R	AD	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	220	21-02-2019	V. N. de Gaia	North	A	Accipitriformes	*A. gentilis*	R	AD	F	Trauma	N	N	N	N	N
CRAS-HVUTAD	221	23-02-2019	Mondim de Basto	North	A	Charadriiformes	*G. gallinago*	M	AD	NA	Trauma	N	N	N	N	NA
CRAS-HVUTAD	222	27-02-2019	Peso da Régua	North	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	223	02-03-2019	Boticas	North	A	Passeriformes	*C. corone*	R	AD	NA	Unknown	N	N	N	N	NA
CRAS-HVUTAD	224	02-03-2019	Vila Real	North	A	Strigiformes	*S. aluco*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	225	03-03-2019	Izeda	North	A	Ciconiiformes	*C. ciconia*	M	AD	F	Dystocia	N	N	N	N	N
CRAS-HVUTAD	227	08-03-2019	Boticas	North	A	Passeriformes	*P. pica*	R	AD	NA	Unknown	N	N	N	N	N
CRAS-HVUTAD	228	15-03-2019	Moimenta da Beira	Centre	A	Charadriiformes	*L. michahellis*	R	AD	F	Weakness	N	N	N	N	N
CRAS-HVUTAD	229	19-03-2019	Gerês	North	A	Accipitriformes	*M. migrans*	M	AD	NA	Unknown	N	N	N	N	N
CRAS-HVUTAD	230	20-03-2019	Borbela	North	A	Accipitriformes	*A. nisus*	R	AD	M	Trauma	N	N	N	N	NA
CRAS-HVUTAD	231	27-03-2019	NA	NA	A	Strigiformes	*S. aluco*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	232	28-03-2019	V. N. Montalegre	North	A	Accipitriformes	*B. buteo*	R	AD	M	Unknown	N	N	N	N	P
CRAS-HVUTAD	233	25-03-2019	PB Gaia	North	A	Accipitriformes	*A. nisus*	R	AD	F	Trauma	N	N	N	N	N
CRAS-HVUTAD	234	18-03-2019	NA	NA	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	235	03-04-2019	Vila Real	North	A	Columbiformes	*S. decaocto*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	237	13-04-2019	Castelo Branco	Centre	A	Accipitriformes	*A. monachus*	R	AD	NA	Trauma	N	N	N	N	P
CRAS-HVUTAD	238	14-04-2019	Torre de Moncorvo	North	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	239	04-05-2019	Vila Real	North	A	Passeriformes	*G. glandarius*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	240	08-05-2019	Lamego	Centre	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	241	08-05-2019	Lamego	Centre	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	242	08-05-2019	Lamego	Centre	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	243	10-05-2019	Miranda do Douro	North	A	Accipitriformes	*H. pennatus*	M	AD	M	Trauma	N	N	N	N	P
CRAS-HVUTAD	244	15-05-2019	Vila Nova de Gaia	North	A	Accipitriformes	*H. pennatus*	M	AD	NA	Trauma	N	N	N	N	P
CRAS-HVUTAD	245	16-05-2019	Miranda do Douro	North	A	Strigiformes	*B. bubo*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	246	26-05-2019	Peso da Régua	North	A	Accipitriformes	*B. buteo*	R	AD	NA	Trauma	N	N	N	N	N
CRAS-HVUTAD	247	26-05-2019	Chaves	North	A	Ciconiiformes	*C. ciconia*	M	JUV	NA	Nestling	N	N	N	N	N
CRAS-HVUTAD	248	26-05-2019	Vinhais	North	A	Ciconiiformes	*C. ciconia*	M	JUV	NA	Nestling	N	N	N	N	N

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Appendix 3 – Continuation.

Centre	Sample	Sample collection day	Locality where was found	Region	Status	Order	Species	Migratory status	Age	Sex	Admission cause	RT-qPCR				WNV Seroneutralization
												WNV	USUV	AOaV-1	IAV	
LxCRAS	300	20-04-2019	Vialonga	South	A	Strigiformes	*T. alba*	R	AD	NA	Unknown	N	N	N	N	N
LxCRAS	301	20-04-2019	Cais do Sodré	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	302	20-04-2019	Seixal	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	303	20-04-2019	Cascais	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	304	20-04-2019	Oeiras	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	305	20-04-2019	V.F. Xira	South	A	Strigiformes	*A. flammeus*	M	AD	NA	Trauma	N	N	N	N	N
LxCRAS	306	20-04-2019	Lisboa	South	A	Charadriiformes	*L. michahellis*	R	AD	NA	Trauma	N	N	N	N	N
LxCRAS	307	20-04-2019	Cascais	South	A	Charadriiformes	*L. michahellis*	R	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	308	20-04-2019	Almada	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	309	20-04-2019	Arrábida	South	A	Falconiformes	*F. peregrinus*	R	AD	NA	Trauma	N	N	N	N	N
LxCRAS	310	20-04-2019	Quinta do Conde	South	A	Anseriformes	*A. platyrhinchos*	M		M	Trauma	N	N	N	N	NA
LxCRAS	311	20-04-2019	Entrecampos	South	A	Anseriformes	*A. aegiptiaca**	R	AD	NA	Trauma	N	N	N	N	N
LxCRAS	312	18-05-2019	Sintra	South	A	Charadriiformes	*L. michahellis*	R	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	313	18-05-2019	Costa da Caparica	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	314	18-05-2019	Paço d'Arcos	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	315	18-05-2019	Cais do sodré	South	A	Charadriiformes	*L. michahellis*	R	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	316	18-05-2019	Costa da Caparica	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	317	18-05-2019	Costa da Caparica	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	318	18-05-2019	Seixal	South	A	Charadriiformes	*L. fuscus*	M	AD	NA	Intoxication	N	N	N	N	N
LxCRAS	319	18-05-2019	Setúbal	South	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
LxCRAS	320	18-05-2019	Ferreira Alentejo	South	A	Strigiformes	*T. alba*	R	AD	M	Trauma	N	N	N	N	N
LxCRAS	321	18-05-2019	Pinhal Novo	South	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
LxCRAS	322	18-05-2019	Charneca caparica	South	A	Strigiformes	*S. aluco*	R	AD	NA	Trauma	N	N	N	N	N
LxCRAS	323	18-05-2019	Ericeira	South	A	Strigiformes	*A. noctua*	R	JUV	NA	Nestling	N	N	N	N	N
LxCRAS	324	18-05-2019	Sintra	South	A	Strigiformes	*A. noctua*	R	AD	NA	Trauma	N	N	N	N	N
LxCRAS	325	18-05-2019	NA	NA	A	Strigiformes	*S. aluco*	R	JUV	NA	Nestling	N	N	N	N	N
LxCRAS	326	18-05-2019	Santarém	South	A	Strigiformes	*S. aluco*	R	AD	NA	Trauma	N	N	N	N	N
LxCRAS	327	18-05-2019	Santarém	South	A	Ciconiiformes	*C. ciconia*	M	AD	NA	Suspicion of intoxication	N	N	N	N	P
LxCRAS	328	18-05-2019	Sintra	South	A	Accipitriformes	*B. buteo*	R	AD	NA	Trauma	N	N	N	N	P
LxCRAS	329	18-05-2019	NA	NA	A	Charadriiformes	*L. fuscus*	M	AD	NA	Paretic syndrome	N	N	N	N	N
LxCRAS	330	18-05-2019	Coruche	South	A	Strigiformes	*T. alba*	R	AD	F	Trauma	N	N	N	N	N
RIAS	331	09-06-2019	Loulé	South	D	Strigiformes	*S. aluco*	R	JUV	F	Nestling	N	N	N	N	NA
RIAS	332	09-06-2019	Mértola	South	D	Strigiformes	*S. aluco*	R	AD	F	Unknown	N	N	N	N	NA
RIAS	333	09-06-2019	Aljezur	South	A	Accipitriformes	*B. buteo*	R	JUV	NA	Nestling	NA	NA	NA	NA	N

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Appendix 4 - Fusion gene "pilot" dataset of class II AOaV-1 used in this study. It contains 132 sequences used to build the tree seen in figure 11 (based in Dimitrov et al. 2019).

Genotypes	Acc. Number	Host	Country	Isolate	Year
I.1.1	AY935490	Chicken	Australia	2_1334	2002
I.1.1	AY935495	Chicken	Australia	99_868_hi	1999
I.1.1	M24693	Chicken	Australia	Queensland	1966
I.1.2.1	EF564816	Redknot	USA	NJ_A_101_1383	2001
I.1.2.1	GQ918280	Black headed gull	Sweden	-	1994
I.1.2.1	KX352834	Gull	Russia	Tyva_14	2014
I.1.2.2	AB465607	Chicken	Japan	Ishi	1962
I.1.2.2	KC503476	Northern pintail	USA	AK_44500_136	2009
I.1.2.2	KC503479	Redpoll	Russia	Nikita_530_FFNK2	2008
I.2	AY965079	Duck	Russia	FarEast_2713	2001
I.2	HG326605	Spur winged goose	Nigeria	NIE08_121	2008
I.2	KC503453	American green winged teal	USA	AK_44493_716	2009
II	AF077761	Chicken	USA	Lasota	1946
II	GU978777	Chicken	USA	TX_GB	1948
II	JN872151	Chicken	USA	Hitchner_B1	1947
III	EF201805	Avian	-	Mukteswar	1940
III	GU182327	Chicken	Pakistan	SPVC_Karachi_1	1974
III	MH996904	Pigeon	Bulgaria	Novo_Selo_1161	1995
IV	AY741404	Fowl	UK	Herts	1933
IV	MH996900	Pullet	Bulgaria	Plovdiv_1153	1959
V.1	JN872189	Parrot	USA	Coast_8278	1982
V.1	JN872194	Chicken	Honduras	498109_15	2007
V.1	JN942027	Fighting cock	Nicaragua	95066_9	2001
V.2	EU518682	Dove	Mexico	Distrito_Federal_462	2004
V.2	EU518684	Chicken	Mexico	Estado_de_Mexico_466	2006
V.2	JQ697744	Chicken	Mexico	NC04_635	2010
VI.2.1.1.1	JX901367	Pigeon	USA	PA_810	2008

Appendix 4 - Continuation.

Genotypes	Acc. Number	Host	Country	Isolate	Year
VI.2.1.1.1	JX901351	pigeon	USA	NJ_721	2007
VI.2.1.1.1	MG018211	ECDO	USA	TX_1185_kidney_26981_3_A	2015
VI.2.1.1.1	EF555096	Human	USA	clone P-F1 fusion protein gene	2007
VI.2.1.1.1	AF503647	Pigeon	Portugal	pigeon/Portugal/8893/98	1998
VI.2.1.1.1	AY471774	Pigeon	Portugal	PPTPI01315	2001
VI.2.1.1.1	AY471775	Pigeon	Portugal	PPTPI99395	1999
VI.2.1.1.1	AY471776	Pigeon	Portugal	PPTPI98388	1998
VI.2.1.1.1	AY471777	Pigeon	Portugal	PPTPI99393	1999
VI.2.1.1.2.1	JX094510	Pigeon	China	sms12	2012
VI.2.1.1.2.1	JX901110	Pigeon	Belgium	248_	1998
VI.2.1.1.2.1	JX486553	Pigeon	China	LHLJ_110813	2011
VI.2.1.1.2.2	KT163262	Pigeon	China	SH_167	2013
VI.2.1.1.2.2	JX901124	Pigeon	Belgium	11_09620	2011
VI.2.1.1.2.2	MG840654.1	Pigeon	China	Ningxia_2068	2016
VI.2.1.1.2.2	KJ544861	Human	Netherlands	NL/Human/2003	2003
VI.1	AF109885	Domestic fowl	Great_Britain	GB1168	1984
VI.1	FJ410145	Pigeon	USA	NY	1984
VI.1	FJ865434	Pigeon	China	S_1	2002
VI.2.2.2	FJ480825	Pigeon	China	PG_JS_1	2005
VI.2.2.2	JX244794	Pigeon	China	100	2008
VI.2.2.2	KJ607163	Pigeon	China	LJS_1	2004
VI.2.2.1	JN872180	Waterfowl	USA	TX_209682	2002
VI.2.2.1	JN872182	Pigeon	USA	12339	1998
VI.2.2.1	JX901312	Pigeon	USA	101	2001
VI.2.1.2	HG326604	Pigeon	Nigeria	NIE09_1898	2009
VI.2.1.2	JX518532	Laughing dove	Kenya	B2_Isiolo	2012
VI.2.1.2	HG424627	Pigeon	Nigeria	NIE13_92	2013
VII.1.1	EF589133	Pheasant	China	98_Guizhou	1998

Appendix 4 - Continuation.

Genotypes	Acc. Number	Host	Country	Isolate	Year
VII.1.1	EF579733	Chicken	China	Shandong_Pyan	2004
VII.1.1	AB853927	Chicken	Japan	Ibaraki_SG106	1999
VII.1.1	KC542905	Chicken	China	Liaoning_1_2009	2009
VII.1.1	KX268351	Chicken	Iran	Behshahr	2015
VII.1.2	AY028995	Fowl	China	A7	1996
VII.1.2	GQ338309	Pigeon	China	18	2003
VII.1.2	DQ227246	Goose	China	Jiangsu_JS02	1999
VII.2	MF622047	Chicken	South_Africa	RBWW_3	2013
VII.2	KU862293	Parakeet	Pakistan	Karachi_AW_1	2014
VII.2	HQ697254	Chicken	Indonesia	Banjarmasin_10	2010
VII.2	KY747479	Chicken	Namibia	5620	2016
VII.2	JN986837	Chicken	Netherlands	152608_ancestral	1993
VIII	AY734534	Chicken	Argentina	Trenque_Lauquen	1970
VIII	FJ751918	Chicken	China	QH1	1979
VIII	JX012096	Chicken	Malaysia	AF2240	1960
IX	AF458009	Chicken	China	FJ_1	1985
IX	FJ436303	Chicken	China	ZJ_1	1986
IX	FJ436302	Chicken	China	F48E8	1948
X	FJ705468	Mottled duck	USA	TX_130	2011
X	KX857716	Redhead	USA	ndv42_AI09_4117	2009
X	FJ705466	Mallard	-	99_376	1999
X	KX857721	Mallard	USA	MN_AI10_3434	2010
XI	HQ266602	Chicken	Madagascar	MG_725	2008
XI	JX518882	Chicken	Madagascar	MGMNJ	2009
XI	JX518884	Chicken	Madagascar	MGS1595T	2011
XII.1	KU594615	Chicken	Peru	Apurimac_50009	2005
XII.1	KU594616	Gamecock	Peru	Lurin_40871	2004
XII.1	KU594618	Chicken	Peru	Arequipa_VFAR_81	2015

Appendix 4 - Continuation.

Genotypes	Acc. Number	Host	Country	Isolate	Year
XII.2	JN627504	Goose	China	GD_12	2011
XII.2	JN627507	Goose	China	GD_1003	2010
XII.2	MF278927	Goose	China	FS_SS_292	2013
XIII.1.1	JN942034	Ostrich	South_Africa	45445_3	1995
XIII.1.1	JN942043	Roller	Tanzania	47385_11	2010
XIII.1.1	MF409241	Chicken	Zambia	Chiwoko	2015
XIII.2.1	GU182323	Chicken	Pakistan	SPVC_Karachi_43	2008
XIII.2.1	GU182331	Chicken	Pakistan	SPVC_Karachi_33_	2007
XIII.2.1	KF113338	Chicken	Pakistan	University_Diagnostic_Lab_12	2010
XIII.2.2	KM056349	Chicken	India	ndv42_gopalpura_4	2013
XIII.2.2	KT734767	Chicken	India	Polashbari	2014
XIII.2.2	KX372707	Chicken	India	Nagpur_3	2011
XIII.1.2	JQ267579	Chicken	Iran	EMM_7	2011
XIII.1.2	JQ267584	Chicken	Iran	EMM_2	2008
XIII.1.2	JQ267585	Chicken	Iran	EMM_1	2008
XIV.1	HF969205	Turkey	Nigeria	NIE09_2071	2009
XIV.1	JN872165	Chicken	Niger	VIR_1377_7	2006
XIV.1	JQ039386	Chicken	Nigeria	VRD08_36	2008
XIV.2	HF969187	Chicken	Nigeria	NIE08_453	2008
XIV.2	HF969210	Chicken	Nigeria	NIE10_139	2011
XIV.2	KY171990	Chicken	Nigeria	KD_TW_03T_N45_720	2009
XVI	JX915242	Chicken	Dominican_Republic	28138_4	1986
XVI	JX915243	Chicken	Mexico	Queretaro_452_1947	1947
XVI	JX186997	Chicken	Dominican_Republic	867	2008
XVII	HF969176	Chicken	Nigeria	NIE10_310	2011
XVII	HF969191	Chicken	Nigeria	NIE08_2042	2009
XVII	HF969194	Chicken	Nigeria	NIE08_2199	2009
XVIII.1	FJ772455	-	Mauritania	1532_14	2006

Appendix 4 - Continuation.

Genotypes	Acc. Number	Host	Country	Isolate	Year
XVIII.1	JF966389	Guinea fowl	Mali	ML038	2007
XVIII.1	JX518885	Chicken	Mali	ML57051T	2010
XVIII.2	HF969218	Chicken	Ivory_Coast	CIV08_42	2007
XVIII.2	HG326600	Village weaver	Ivory_Coast	CIV08_32	2006
XVIII.2	JX518886	Chicken	Mali	ML57072T	2010
XIX	FJ705456	Cormorant	USA	MN_92_40140	1992
XIX	JN942024	Cormorant	USA	WI_272409	2003
XIX	KC433530	Cormorant	USA	FL_41105	2012
XX	AB853928	Chicken	Japan	Ibaraki_SM87	1987
XX	AF458016	Chicken	China	ZhJ_2	1986
XX	KY042142	Quail	Korea	88_M	1988
XXI	KC205479	Chicken	Ethiopia	ETHMG1C	2011
XXI.2	JN638234	Dove	Italy	11RS98_102VIR	2011
XXI.2	KU377533	Turtle dove	Italy	10VIR7155	2010
XXI.2	KU377535	Turtle dove	Italy	12VIR1876_1	2012
XXI.2	KX831612	Pigeon	Portugal	16919-15	2015
XXI.2	KX831613	Pigeon	Portugal	17991-15	2015
XXI.2	KX831614	Pigeon	Portugal	19095- 15	2015
XXI.2	-	Eurasian collared dove	Portugal	Sample 118	2019
XXI.2	-	Eurasian collared dove	Portugal	Sample 120	2019
XXI.1.2	KU862298	Pigeon	Pakistan	Lahore_AW_2	2015
XXI.1.2	KY042135	Pigeon	Pakistan	22A	2015
XXI.1.2	KY042141	Pigeon	Pakistan	Jallo_Lahore_221B	2016
XXI.1.1	JF824032	pigeon	Russia	Vladimir_687	2005
XXI.1.1	KY042136	Pigeon	Pakistan	Lahore_125	2015
XXI.1.1	KY042132	Pigeon	Egypt	73_OP_G29	2015

Appendix 5 - Fusion gene dataset of class II genotype XXI AOaV-1 used in this study (based in Dimitrov et al. 2019). It contains 56 sequences used to build the sub-tree seen in figure 12, together with rooting sequences JX915243 and Z12111.

Genotypes	Acc. Number	Host	Country	Isolate	Year
XVI	JX915243	Chicken	Mexico	Queretaro_452_1947	1947
UNCL	Z12111	Chicken	Great Britain	Warwick_ancestral	1966
XXI	KC205475	Chicken	Ethiopia	ETH10065	2011
XXI	KC205476	Chicken	Ethiopia	_ETH_10073	2011
XXI	KC205477	Chicken	Ethiopia	ETH8755	2011
XXI	KC205478	Chicken	Ethiopia	ETHAN01	2011
XXI	KC205479	Chicken	Ethiopia	ETHMG1C	2011
XXI	KJ958913	Chicken	Ethiopia	13VIR3936_1	2012
XXI	KJ958914	Chicken	Ethiopia	13VIR3936_27	2012
XXI.1.1	JF824013	Pigeon	Russia	Kemerovo_0267	2009
XXI.1.1	JF824032	Pigeon	Russia	Vladimir_687	2005
XXI.1.1	KJ914671	Pigeon	Ukraine	Dnipropetrovsk_1_18_11	2011
XXI.1.1	KJ914672	Pigeon	Ukraine	Ukromne_3_26_11	2011
XXI.1.1	KT962979	Pigeon	Russia	Altai_777	2010
XXI.1.1	KT965727	Pigeon	Kazakhstan	EKO_15	2014
XXI.1.1	KT965728	Pigeon	Kazakhstan	Zhambyl_32	2014
XXI.1.1	KX352835	mallard	Russia	Amur_264	2009
XXI.1.1	KY042127	pigeon	Ukraine	Kharkiv_23_01_967	2013
XXI.1.1	KY042128	Pigeon	Ukraine	Doneck_3	2007
XXI.1.1	KY042129	Pigeon	Egypt	11_CL_G1_	2015
XXI.1.1	KY042130	Pigeon	Egypt	44_CL_G24_	2015
XXI.1.1	KY042131	Pigeon	Egypt	56_CL_G25_	2015
XXI.1.1	KY042132	Pigeon	Egypt	73_OP_G29_	2015
XXI.1.1	KY042134	Pigeon	Egypt	84_OP_G31_	2015
XXI.1.1	KY042136	Pigeon	Pakistan	Lahore_125	2015
XXI.1.1	KY042137	Pigeon	Pakistan	Jhang_115	2015
XXI.1.1	KY042138	Pigeon	Pakistan	Lahore_126	2015

Appendix 5 - Continuation.

Genotypes	Acc. Number	Host	Country	Isolate	Year
XXI.1.1	KY042139	Pigeon	Pakistan	Lahore_146	2016
XXI.1.1	JQ039385	dove	Nigeria	dove_VRD07_163	2007
XXI.1.1	MH717070	Pigeon	Pakistan	AJK_AW_p54	2018
XXI.1.1	MH996953	Pigeon	Nigeria	Kazaure_VRD231_42	2007
XXI.1.1	MK005973	Pigeon	Egypt	Souqal_Cairo_39_L_G23_1105	2015
XXI.1.2	KU862297	Pigeon	Pakistan	Lahore_AW_1	2014
XXI.1.2	KU862298	Pigeon	Pakistan	Lahore_AW_2	2015
XXI.1.2	KU885949	Pigeon	Pakistan	MZS_UVAS	2014
XXI.1.2	KX236100	Pigeon	Pakistan	21A	2015
XXI.1.2	KX236101	Pigeon	Pakistan	25A	2015
XXI.1.2	KY042135	Pigeon	Pakistan	22A	2015
XXI.1.2	KY042140	Pigeon	Pakistan	Jallo_Lahore_221A	2016
XXI.1.2	KY042141	Pigeon	Pakistan	Jallo_Lahore_221B	2016
XXI.1.2	MH717071	Pigeon	Pakistan	AJK_AW_p51	2018
XXI.1.2	MH717072	Pigeon	Pakistan	AJK_AW_p52	2018
XXI.1.2	MH717073	Pigeon	Pakistan	AJK_AW_p53	2018
XXI.2	HG424625	Pigeon	Nigeria	NIE13_005	2013
XXI.2	JN638234	Dove	Italy	11RS98_102VIR	2011
XXI.2	JN638235	Dove	Italy	11RS100_104VIR	2011
XXI.2	JN638236	Dove	Italy	10RS6171_7154VIR	2010
XXI.2	KU377533	Turtle dove	Italy	10VIR7155	2010
XXI.2	KU377535	Turtle dove	Italy	12VIR1876_1	2012
XXI.2	KU377536	Turte dove	Italy	12VIR604	2012
XXI.2	MG456676	Collared dove	Iran	-	2014
XXI.2	MH044693	Pigeon	Iran	Konarak_Barin	2017
XXI.2	MH377298	Eurasian collared dove	Israel	PHL264746	2010
XXI.2	KX831612	Pigeon	Portugal	16919-15	2015
XXI.2	KX831613	Pigeon	Portugal	17991-15	2015
XXI.2	KX831614	Pigeon	Portugal	19095- 15	2015
XXI.2	-	Eurasian collared dove	Portugal	Sample 118	2019
XXI.2	-	Eurasian collared dove	Portugal	Sample 120	2019

Appendix 6 – EURING age codes. Based on EURING 2020.

0	Age unknown.
1	Pullus: nestling or chick, unable to fly freely.
2	Full-grown: adult bird, able to fly freely. Age unknown.
3	1st year: adult bird that hatched in the breeding season of this calendar year.
4	After 1st year: adult bird hatched before this calendar year. Year of hatchling unknown.
5	2nd year: adult bird that hatched in the last calendar year. It is it second calendar year.
6	After 2nd year: adult bird hatched before the last calendar year. Year of hatchling unknown.
7	3rd year: adult bird that hatched two calendar years before. It is it third calendar year.
8	After 3rd year: adult bird hatched more than three calendar years ago. Age of hatchling unknown.
9	4th year: adult bird that hatched three calendar years before. It is it fourth calendar year.
A	After 4th year: adult bird that hatched more than four calendar years ago. Age of hatchling unknown.
B	5th year: adult bird that hatched four calendar years before. It is it fifth calendar year.
C, D, E, F, …	Onwards, with the same sequence

Appendix 7 – Fischer's exact test to access association between bird orders and outcome in AOaV-1 molecular test.

Factor	Fisher's exact test
Accipitriformes	OR = 0, CI 95%: 0-18.49; (p=1)
Anseriformes	OR = 0, CI 95%: 0-91.77; (p=1)
Apodiformes	OR = 0, CI 95%: 0-108.83; (p=1)
Charadriiformes	OR = 0, CI 95%: 0-15.80; (p=1)
Ciconiiformes	OR = 0, CI 95%: 0-91.77; (p=1)
Columbiformes	OR = Inf, CI 95%: 6.79-Inf; (p<0.01)
Falconiformes	OR = 0, CI 95%: 0-91.77; (p=1)
Gruiformes	OR = 0, CI 95%: 0-133.38; (p=1)
Passeriformes	OR = 0, CI 95%: 0-34.37; (p=1)
Strigiformes	OR = 0, CI 95%: 0-9.73; (p=1)
Suliformes	OR = 0, CI 95%: 0-39.55; (p=1)

Appendix 8 – Fischer's exact test to access association between bird species and outcome in AOaV-1 molecular test

Bird species	Fisher's exact test
Accipiter gentilis	OR = 0, CI 95%: 0-1710; (p=1)
Accipiter nisus	OR = 0, CI 95%: 0-;171.76 (p=1)
*Acridotheres cristatellus**	OR = 0, CI 95%: 0-1710; (p=1)
Aegypius monachus	OR = 0, CI 95%: 0-1710; (p=1)
*Alopochen aegyptiaca**	OR = 0, CI 95%: 0-1710; (p=1)
Anas platyrynchos	OR = 0, CI 95%: 0-1710; (p=1)
Apus apus	OR = 0, CI 95%: 0-401.80; (p=1)
Apus pallidus	OR = 0, CI 95%: 0-171.76; (p=1)
Asio flammeus	OR = 0, CI 95%: 0-401.80; (p=1)
Asio otus	OR = 0, CI 95%: 0-1710; (p=1)
Athene noctua	OR = 0, CI 95%: 0-34.37; (p=1)
Bubo bubo	OR = 0, CI 95%: 0-240.51; (p=1)
Buteo buteo	OR = 0, CI 95%: 0-39.55; (p=1)
Chroicocephalus ridibundus	OR = 0, CI 95%: 0-401.80; (p=1)
Ciconia ciconia	OR = 0, CI 95%: 0-91.77; (p=1)
Circaetus gallicus	OR = 0, CI 95%: 0-1710; (p=1)
Corvus corax	OR = 0, CI 95%: 0-1710; (p=1)
Corvus corone	OR = 0, CI 95%: 0-240.51; (p=1)
Erithacus rubecula	OR = 0, CI 95%: 0-1710; (p=1)
Falco naumanni	OR = 0, CI 95%: 0-1710; (p=1)
Falco peregrinus	OR = 0, CI 95%: 0-1710; (p=1)
Falco tinnunculus	OR = 0, CI 95%: 0-133.38; (p=1)
Fringilla coelebs	OR = 0, CI 95%: 0-1710; (p=1)
Fulica atra	OR = 0, CI 95%: 0-171.76; (p=1)
Gallinago gallinago	OR = 0, CI 95%: 0-1710; (p=1)
Gallinula chloropus	OR = 0, CI 95%: 0-1710; (p=1)
Garrulus glandarius	OR = 0, CI 95%: 0-240.51; (p=1)
Hieraaetus pennatus	OR = 0, CI 95%: 0-171.76; (p=1)
Hirundo rustica	OR = 0, CI 95%: 0-1710; (p=1)
Larus audouinii	OR = 0, CI 95%: 0-1710; (p=1)
Larus fuscus	OR = 0, CI 95%: 0-42.71; (p=1)
Larus michahellis	OR = 0, CI 95%: 0-39.55; (p=1)
Mareca strepera	OR = 0, CI 95%: 0-1710; (p=1)
Milvus migrans	OR = 0, CI 95%: 0-401.80; (p=1)
Morus bassanus	OR = 0, CI 95%: 0-39.55; (p=1)
Pernis apivorus	OR = 0, CI 95%: 0-1710; (p=1)

Appendix 8 - Continuation.

Bird species	Fisher's exact test
Pica pica	OR = 0, CI 95%: 0-1710; (p=1)
Spatula clypeata	OR = 0, CI 95%: 0-171.76; (p=1)
Streptopelia decaocto	OR = Inf, CI 95%. 6.79-Inf; (p<0.01)
Strix aluco	OR = 0, CI 95%: 0-28.58; (p=1)
Sturnus unicolor	OR = 0, CI 95%: 0-1710; (p=1)
Sylvia atricapila	OR = 0, CI 95%: 0-1710; (p=1)
*Taeniopygia guttata**	OR = 0, CI 95%: 0-1710; (p=1)
Turdus merula	OR = 0, CI 95%: 0-401.80; (p=1)
Tyto alba	OR = 0, CI 95%: 0-133.38; (p=1)

Appendix 9 - Fischer's exact test to access association between bird order and outcome in WNV serological test.

Bird order	Fisher's exact test
Accipitriformes	OR = 35.85, CI 95%: 4.76-840.82; (p<0.001)
Anseriformes	OR = 0, CI 95%: 0-119.7; (p=1)
Charadriiformes	OR = 0, CI 95%: 0-1.55; (p=0.1)
Ciconiiformes	OR = 2.19, CI 95%: 0.08-22.31; (p=0.45)
Columbiformes	OR = 0, CI 95%: 0-119.7; (p=1)
Falconiformes	OR = 0, CI 95%: 0-119.7; (p=1)
Passeriformes	OR = 0, CI 95%: 0-11.29; (p=1)
Pelecaniformes	OR = 0, CI 95%: 0-119.7; (p=1)
Strigiformes	OR = 0, CI 95%: 0-0.76; (p<0.05)

Appendix 10 - Fischer's exact test to access association between bird species and outcome in WNV serological test.

Bird species	Fisher's exact test
Accipiter gentilis	OR = 0, CI 95%: 0-11.29; (p=1)
Accipiter nisus	OR = 3.31, CI 95%: 0.11-46.22; (p=0.36)
Aegypius monachus	OR = Inf, CI 95%: 0.33-Inf; (p=0.14)
Alopochen aegyptiaca	OR = 0, CI 95%: 0-119.7; (p=1)
Ardea cinerea	OR = 0, CI 95%: 0-119.7; (p=1)
Asio flammeus	OR = 0, CI 95%: 0-119.7; (p=1)
Athene noctua	OR = 0, CI 95%: 0-7.36; (p=1)
Bubo bubo	OR = 0, CI 95%: 0-119.7; (p=1)
Buteo buteo	OR 9.31, CI 95%: 1.80-49.78; (p<0.05)
Ciconia ciconia	OR = 2.19, CI 95%: 0.08-22.31; (p=0.45)
Circaetus gallicus	OR = Inf, CI 95%: 0.33-Inf; (p=0.14)
Corvus corone	OR = 0, CI 95%: 0-119.7; (p=1)

Appendix 10 - Continuation.

Bird species	Fisher's exact test
Falco peregrinus	OR = 0, CI 95%: 0-119.7; (p=1)
Garrulus glandarius	OR = 0, CI 95%: 0-119.7; (p=1)
Hieraaetus pennatus	OR = Inf, CI 95%: 1.92-Inf; (p<0.05)
Larus fuscus	OR = 0, CI 95%: 0-2.07; (p=0.34)
Larus michahellis	OR = 0, CI 95%: 0-6.09; (p=1)
Milvus migrans	OR = 0, CI 95%: 0-119.7; (p=1)
Milvus milvus	OR = 0, CI 95%: 0-22.56; (p=1)
Pica pica	OR = 0, CI 95%: 0-119.7; (p=1)
Streptopelia decaocto	OR = 0, CI 95%: 0-119.7; (p=1)
Strix aluco	OR = 0, CI 95%: 0-1.55; (p=0.1)
Tyto alba	OR = 0, CI 95%: 0-11.29; (p=1)